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13. ABSTRACT (Maximum 200 Words) The present study is a final report of the project. During the project we developed iodine formulation proved to be efficacious against SM in the guinea pig skin model at intervals of 15 and 30 min between exposure and treatment. Incorporation of the antiinflammatory drug piroxicam and the steroidal antiinflammatory agent clobetasol, caused the formulation to protect at intervals of 45 and 60 min in the mouse ear swelling model and at 45 min in the guinea pig skin irritation test. Introduction of ethanol to the formulation caused the preparation to be efficacious in the pig model; 20 min interval between exposure and treatment caused significant protection. Longer intervals will be tested in the near future. The topical preparation was also effective against heat- and hydrofluoric acid-induced skin burns. Toxicokinetic studies with male, fur-covered and hairless guinea pigs showed that SM disappeared from the skin 60 min after exposure whereas in the female, fur-covered guinea pig SM disappeared after 3 hours. Sodium hypochlorite inactivated SM in vitro but not in situ, namely, no significant inactivation by decontaminant applied on SM-exposed skin was observed. Iodine caused reduction in skin collagenase induced by mechlorethamine. COX2-deficient mice were less affected by SM than COX1-deficient and wild type mice. Iodine was also effective against dermally exposed organophosphate (paraoxon). The present study demonstrated that iodine induced skin protective peptides against noxious stimuli. Four peptides were identified and synthesized, of which two had protective activity when injected together with proteinase inhibitors to prevent degradation. Synthesis of N-methylated analogs produced metabolically stable peptides with protective activity against SM-induced skin lesions. Newly synthesized analogs showed protection in the mouse model when injected iv together with proinflammatory chemokine antibodies. It is planned to further develop metabolically stable, pharmacologically active peptides and to determine the optimal conditions for their beneficial activity.				
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INTRODUCTION

Sulfur mustard [$\text{Cl-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-Cl}$] is a highly potent alkylating agent and severely cytotoxic vesicant. Topical exposure to SM results in erythema appears within hours of intoxication followed by edema, blistering and ulceration observed within tens of hours following poisoning (1-9). The devastating effect of SM was demonstrated in several conflicts in this century emphasizing the great need of an efficient pharmacological antidote against mustard gas toxicity. Numerous compounds have been tested for their antidotal activity against SM, however, they were too weak to be used as protectants against the vesicant. The main task of the present research was to develop a topical preparation for post-exposure treatment of SM-exposed individuals. This final report describes the main data of previous reports and the recent improvements obtained in the last year of project. Additional task of the present research was to investigate the molecular mechanism of the protective activity of the newly developed preparation.

BODY

Background

Our preliminary studies have shown that povidone iodine ointment protects guinea pig skin against SM (10-16) Gross and histopathological findings showed that application of povidone iodine 20 min or less following exposure to the vesicant resulted in marked protection. The shorter interval between exposure and treatment the better protection was achieved. We invested an intensive effort in developing a better iodine formulation for improving its protective properties. We found that the basic component for solving iodine is tetraglycol. Under these circumstances iodine does not precipitate in presence

of up to 50% water (17). This iodine formulation possesses much stronger protective activity than the previously used commercial povidone iodine. Post exposure treatment with this preparation resulted in significant protection against SM-induced skin lesions in the haired guinea pig model at intervals of 15 and 30 min, and to a lesser extent at 45 and 60 min, between exposure and treatment (phase I study). Later studies further improved the formulation and significant protection was achieved at 45 and 60 min interval between exposure and treatment (phase II study). Since the two phases were different in their experimental procedure, a detailed procedure will be described for each phase.

EXPERIMENTAL PROCEDURE (phase I study)

Iodine formulations. Formulation A) 2% iodine (Merck) dissolved in tetraglycol (TG) : water 1:1 (liquid formulation); Vehicle A) TG : water 1:1; Formulation B) 2% I₂, 2.4% NaI, in 45.6% ethanol, 50% water (liquid formulation); Vehicle B) 50% ethanol, 50% water; Formulation C) 2% iodine, 50% TG, 40% poloxomer 407, 8% water (ointment formulation).

Experimental procedure. Backs of haired guinea pigs were shaved 24 hours prior to the experiment. The animals were anesthetized by 30 mg/kg sodium pentobarbital ip. Backs were cleaned with wet soft white paper and allowed to dry. Six sites on each back, three on each side, were exposed to 1µl (1.2 mg) SM. One ml of iodine formulation A was applied on three exposure sites of each animal, while the other three SM-exposed sites remained untreated. Iodine was applied into wells constructed by cutting a plastic tube cover (inner diameter of 1.7 cm) to form an open-ended cylindrical well. A thin layer of commercial silicon sealing ointment was applied to the edge of the well attached to the back of the animal so that liquid inside did not leak out. The well was exposed to SM,

and iodine formulation A was applied 15 and 30 min after SM exposure. In an additional series of experiments, the intervals between exposure and treatment were increased to 45 and 60 min. Unless otherwise indicated, the iodine preparation was left on the skin for 2 hours. At the end of the procedure the liquid iodine was aspirated out and the well removed from the skin. Animals were sacrificed using 100 mg/kg sodium pentobarbital ip. Their backs were photographed alongside a ruler using a Kodak 260 digital camera. The ulceration area of each exposure site was assessed grossly. Skin specimens were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5-6 mm, and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

Effect of lidocaine on the protective effect of iodine. Guinea pigs were shaved and anesthetized with sodium pentobarbital. The tested skin area was anesthetized locally with 0.1 ml of 2% lidocaine injected sc about 1 cm laterally to each site of exposure so that each animal received 6 injections. For comparison, control animals were injected with saline. Twenty min later six sites on each animal back were exposed to 1.2 mg neat (1 μ l) SM. After additional 15 min a thin layer of 50 mg of iodine formulation C ointment was applied (well system was not used) on three out of the six exposure sites of each animal while the other three exposure sites of the animal remained untreated. Lidocaine or saline treatment was repeated 2 h after the first injections. The ulcerated area was measured 7 days after exposure. Results are expressed as mean \pm SE using the Mann-Whitney two-tailed test for statistical evaluation of the differences between the control (SM only) and iodine treatment of the lidocaine-injected animals and, the control and iodine treatment of the saline-injected animals.

Determination of the effect of iodine on chemical inactivation of SM *in situ*. Wells were constructed on the backs of pentobarbital-anesthetized guinea pigs as described previously. SM (1µl neat liquid) was applied in the center of each well followed 10 min later by the addition of 1 ml of iodine formulation A or vehicle A. After additional 20 min, the entire iodine solution was aspirated out and extracted with 1 ml of dichloromethane (Frutarum, Haifa, Israel) by rough-mixing for 1 min. The organic phase was subjected to SM analysis. While animals were anesthetized SM in the skin surrounded by the well was extracted by the addition of 1 ml of dichloromethane into the well. To obtain maximal extraction from the skin, the organic solvent was pipetted up and down 3-4 times, then subjected to SM analysis.

As will be shown in the Results section, low SM levels were found in the skin, while both the iodine and vehicle extracts were below the detection level. Therefore, we employed an additional iodine formulation, B, that had protecting properties, although weaker than those of TG-containing preparations (data not shown). The time schedule was extended in order to determine if after a long period of incubation, iodine would affect SM levels. One ml of iodine formulation B or vehicle B was added to the well 20 min after SM exposure. The iodine solution was aspirated out 2 h later and extracted with dichloromethane. The skin was also extracted as described above. SM in dichloromethane samples was quantified by gas chromatography/mass spectrometry (GC/MS) analysis using appropriate standards. Each specimen (5µl) was injected into an HP 5980 II gas chromatograph system equipped with an electron ionizing detector, HT1 30m capillary column and HP 5971A MS detector. The elution method entailed starting at 100°C for 2 min then increasing the temperature at the rate of 10°C/min up to 200°C. To test the pure effect of iodine on SM, analysis of iodine treatment was compared to that obtained by the appropriate iodine vehicle.

Histopathological evaluation. Each skin section was evaluated in a blinded way, without knowing the identity of the treatment group, and scored for histopathological changes. The reactive and inflammatory changes in the epidermis and dermis were assigned a severity grade of 0-4 representing unremarkable, minimal, mild, moderate and marked changes, respectively. Epidermal parameters included: microblister formation, ulceration, necrosis, crust formation, hyperkeratosis, and acanthosis (epidermal hyperplasia). Dermal parameters included: hemorrhage, inflammation (acute and subacute), necrosis and fibrosis. In addition, the area of epidermal acanthosis was estimated using the grade of 0-6 as follows: Grade 0 - No acanthosis; 1 - Less than 1/3 of the epidermal area acanthotic, the remainder necrotic; 2 - About 1/3 acanthotic, the remainder necrotic; 3 - About 1/2 acanthotic, the remainder necrotic; 4 - Less than 2/3 (but more than 1/2) acanthotic, the remainder necrotic; 5 - More than 2/3 acanthotic, the remainder necrotic; 6 - Diffuse acanthosis (entire epidermal area acanthotic).

Quantitative evaluation: To quantify the protective effect of the iodine formulation against SM, we evaluated 57 skin specimens exposed to SM only, 9 treated with iodine 15 min after exposure, and an additional 18 with a 30 min interval between exposure and iodine treatment. Skin specimens were collected 2 days after treatment. In an additional series of experiments with 45 (n=15) and 60 (n=15) min intervals between exposure and treatment and a concurrent control group (SM only, n=53), animals were sacrificed 6 days after treatment. Results are expressed as the mean \pm SE using the Kruskal Wallis test and Dunnett's multiple comparison post test for statistical comparison of the effects of SM and iodine at different time intervals between exposure and treatment.

RESULTS

Gross pathology findings. The macroscopic appearance of a guinea pig exposed to SM and treated topically with iodine 30 min later (Fig. 1) demonstrates the protective effect of iodine in 2 out of 3 treated sites. Evaluation of a series of experiments revealed statistically significant reductions of 91% and 84% in the grossly ulcerated area at intervals of 15 and 30 min between exposure and treatment, respectively (Fig. 2). In order to rule out dilution effect of SM, we show that the iodine vehicle formulation had a weak protective effect (32% reduction in ulceration) which was not statistically different from the exposed, untreated sites.

Histopathological findings. The histopathological findings are quantified for various treatment groups in Figures 3 and 4. At the interval of 15 min between exposure and treatment, a statistically significant reduction of 48%, 50% and 55% was observed in dermal parameters indicative of acute tissue damage such as acute inflammation, hemorrhage and necrosis, respectively (Fig. 3, lower profile). Moreover, the epidermal healing markers, acanthosis and hyperkeratosis, were significantly increased by 67% and 72%, respectively (Fig. 3, upper profile). These healing parameters are the most reliable histological proof for the effectiveness of iodine. At the longer interval of 30 min between SM exposure and iodine treatment, a significant degree of protection was conferred, albeit to a lesser extent than that observed in the shorter interval. Although the epidermal healing markers were not elevated, the parameters indicative of acute tissue damage such as subepidermal microblister formation, epidermal ulceration, and dermal markers including acute inflammation, hemorrhage and necrosis were significantly reduced

by 35%, 67%, 43%, 39% and 45%, respectively (Fig. 3). Iodine had weaker protective activity at the 45-min interval between exposure and treatment (Fig. 4). A degree of protection was expressed by a statistically significant reduction in dermal subacute inflammation, subepidermal microblister formation, and epidermal ulceration; whereas, the regenerative marker, epidermal acanthosis, was significantly increased. At the interval of 60 min between exposure and treatment, iodine was less effective; nevertheless, a significant reduction in subepidermal microblisters and an increase in the acanthotic area were observed even at this interval.

***In vivo* effect of iodine on chemical inactivation of SM.** The primary question regarding the mechanism of the protective effect of iodine is whether SM is chemically inactivated by iodine in the skin of the living guinea pig. In order to address this issue animals, after exposure to SM, were treated with liquid iodine preparations or their vehicles in the well system described in the Materials and Methods. SM extracted from the skin and from iodine preparations or their vehicle was quantitatively determined by GC/MS analysis (Fig. 5). Two iodine formulations and their vehicles were tested. A slight iodine-induced SM reduction of 36% and 22%, in comparison to the vehicles, was observed after vesicant extraction from skin treated with formulations A and B, respectively (Fig. 5). No iodine-induced SM inactivation was observed after extraction of SM from the liquid iodine preparations (Fig. 5).

Effect of lidocaine on the protective effect of iodine. Because previous studies have shown that neuronal activity is involved in the evolution of skin damage

caused by chemical irritants (Veronesi *et al.*, 1995), we tested the effect of the local anesthetic lidocaine on both skin toxicity of SM and the protective effect of iodine (Fig. 6). Local anesthesia affected neither the area of skin ulceration nor the protective benefits of iodine, when compared to saline-treated controls.

Our first objective was to further improve the iodine preparation, namely, to extend the interval between exposure and iodine treatment in order to obtain a topical preparation that will be efficacious in the battlefield conditions

The experimental approach

The evolution of SM-induced skin lesion involves a variety of inflammatory processes and production of proinflammatory factors (25-31). The dermal infiltration of polymorphnuclear cells upon SM exposure (18, 30) further supports the involvement of inflammation in skin irritation induced by this vesicant. These findings stimulated the introduction of non-steroidal antiinflammatory drugs (NSAID) into a series of compounds to be tested against SM-induced skin lesions. Studies by Buxton *et al.* (32), Babin *et al.* (33) and Zhang *et al.* (34) have shown the protective activity of topical and parenteral treatments with the NSAID including indomethacin and olvanyl against the skin toxicity of SM. Thus, it was suggested to combine both NSAID and iodine into the topical preparation. Although these agents had some protective effect, they were not efficacious enough to be used as antidotes. A reasonable explanation for their limited beneficial activity by parenteral injection may stem from the relatively low concentrations at the target tissue, namely, the skin. In order to increase the local skin concentration of the drugs we developed an iodine formulation that contained a combination of both NSAID and topical steroid. From the various NSAID tested by us, piroxicam and clobetasol possessed the most potent counter-irritating activity.

EXPERIMENTAL PROCEDURE (phase II study)**Mouse ear swelling test**

Male ICR mice (~25g) were anesthetized by pentobarbital sodium 60mg/kg ip (0.1ml/25g BW of 1.5% solution) and placed on their abdominal side. Anesthesia was maintained by 0.03ml/25g BW whenever needed. The outer side of the ear was exposed to 0.317 mg SM (5µl of 1:20 dilution in dichloromethane). The protective formulation, termed N66 (10% povidone iodine, 2% piroxicam, 0.5% clobetasol in 75% tetraglycol and 25% water) was applied 20, 30, 45 and 60 min after SM exposure by the following procedure: 3 layers gauze pad (about 0.7X0.7 cm) soaked in N66 were placed on the ear so the entire site of exposure was applied with the liquid formulation. The pad was left on the skin for 2 hours; during that time the gauze pad was reapplied with N-66 whenever it became dry. In the end of the procedure (2 hours after N-66 application) the pad was removed. Mouse ear thickness was measured 24 and 48 hours after exposure using micrometer (Model PK-0505, Mitutoyo Corporation, Japan). Edema was assessed by the difference between ear thickness measured after and prior to exposure. Animals were sacrificed 48 hours after exposure. Animals were sacrificed using 100 mg/kg sodium pentobarbital ip. Ear specimens were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5-6 mm, and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

Guinea pig skin irritation test

Backs of haired guinea pigs were shaved 24 hours prior to the experiment. The animals were anesthetized by 30 mg/kg sodium pentobarbital ip. Backs were cleaned with wet soft white paper and allowed to dry. Six sites on each back, three on each side, were

exposed to 1µl (1.27 mg) neat SM. Forty five min later the protective preparation N66 (10% povidone iodine, 2% piroxicam, 0.5% clobetasol in 75% tetraglycol and 25% water) was applied on three exposure sites of each animal, while the other three SM-exposed sites remained untreated. The application procedure was carried out as follows: 3 layers gauze pad (about 1x1 cm) soaked in N66 were placed on each exposed area so the entire site of exposure was applied with the liquid formulation. The pad was left on the skin for 2 hours; during that time the gauze pad was reapplied with N66 whenever it became dry. In the end of the procedure (2 hours after N66 application) the pad was removed. Since N66 had some protective effect on adjacent, untreated SM-exposed skin areas, a series of experiments was carried out in which guinea pigs were exposed to SM only without protective treatment. Animals were sacrificed 7 days after exposure using 100 mg/kg sodium pentobarbital ip. Their backs were photographed alongside a ruler using a Kodak 260 digital camera. The ulceration area of each exposure site was assessed grossly. Skin specimens were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5-6 mm, and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

Histopathological evaluation. Histopathological evaluation and its quantification were carried out as described in Phase I.

RESULTS

Mouse ear edema

Gross pathology: The protective effect of the combination of povidone iodine, clobetasol and piroxicam (N66) against SM-induced ear swelling is demonstrated in Fig. 7. Twenty four hours after treatment there was a statistically significant reduction of 69%, 50%, 49% and 38% in

SM toxicity, while 48 hours after treatment there was 43%, 47%, 44% and 36% reduction in mouse edema at time intervals of 20, 30, 45 and 60 min between exposure and treatment, respectively. The vehicle had also some beneficial effect of about 30% reduction 24 hours after treatment and almost no effect after additional 24 hours.

Histopathology: Histopathological evaluation of mouse skin 2 days after exposure to SM showed epidermal ulceration covered by encrustation and epidermal necrosis. However, treatment of the SM-exposed ear with N66 20 min after intoxication strongly protected the skin and prevented the evolution of the burn, resulting in intact skin devoid of lesions. N66 was markedly efficacious at longer intervals between exposure and treatment; epidermal acanthosis covered with encrustation was noted at 30 min interval and at 45 min interval there was epidermal necrosis and at 60 min also dermal hemorrhage appeared. Nevertheless, these lesions were less severe than those of the SM-exposed without N66 treatment, namely, at these relatively long time intervals our formulation had protective effect, although to a lesser extent than that observed in the shorter time intervals between exposure and treatment.

Quantification of the protective effect of N66 against SM in the mouse ear model is shown in Fig. 9 (epidermal parameters) and 10 (dermal parameters). In general, a more pronounced protective effect was observed when N66 was applied 20 min after exposure, nevertheless, longer intervals also showed statistically significant protection. N66 reduced epidermal ulceration by 74%, 58%, 45% and 58% at time intervals between exposure and treatment of 20, 30, 45 and 60 min, respectively (Fig. 9). Similar effect was observed with epidermal necrosis and encrustation; N66 caused reduction of 54%, 34%, 26% and 31% in the former and 62%, 43%, 24% and 33% in the latter, at time intervals of 20, 30, 45 and 60 min, respectively, between exposure and treatment. However, the healing marker, grade of acanthotic area, showed dramatic increase of 39.6, 25.3, 20.9 and 22 fold upon treatment with N66 20, 30, 45, and 60 min after exposure, respectively (Fig. 9). The dermal parameters (Fig.

10) were also markedly influenced by N66 while, as for the epidermal parameters, the 20 min interval had the most efficacious protection. Acute inflammation was reduced by 63%, 34%, 34% and 38%, hemorrhage showed similar results and dermal necrosis was reduced by 80%, 54%, 54% and 59%, at time intervals of 20, 30, 45 and 60 min, respectively.

Guinea pig skin irritation

Gross pathology: The macroscopic lesions of SM-exposed guinea pig skin and the protective effect of N66 are illustrated in Fig. 11 and quantified in Fig. 12. Exposure to 1 µl neat SM resulted in severe skin ulcerations in all sites of exposure (Fig. 11, lower photos). However, topical treatment with N66 45 min after exposure, significantly reduced SM skin toxicity (Fig. 11, upper photos), namely, no ulceration was observed in 2 out of 3 treated sites, whereas the third (Fig. 11 upper photos, left lower site). Interestingly, the untreated, control sites (Fig. 11 upper photos, right sites) also showed some protection expressed by smaller ulceration areas than those of the pure controls. This means that, in addition to the N66-treated sites, adjacent zones may also be protected against SM. Quantification of the ulcerated (Fig. 12) areas shows the same trend, namely, N66-treatment 45 min after SM exposure caused statistically significant reduction of 75% in ulceration area as compared to pure control, whereas control (skin treated in adjacent sites with N66) showed reduction of 34% in the ulceration area.

Histopathology: Histopathological evaluation of guinea pig skin 7 days after exposure to 1 µl neat SM only (pure control, the animals were not treated at all with N66, showed the presence of epidermal acanthosis and, epidermal ulceration covered by encrustation. However, treatment of guinea pig skin 45 min after SM exposure significantly reduced the skin lesion as expressed by increase of the healing marker epidermal acanthosis accompanied with encrustation. No ulceration was observed. Nevertheless, SM-exposed skin that adjacent sites

were treated with N66 (control) showed the presence of epidermal acanthosis, epidermal encrustation and subepidermal microblister formation, but no ulceration.

Quantification of the protective effect of N66 against SM in the guinea pig skin model is shown in Fig. 14. Topical treatment with the preparation 45 min after exposure resulted in statistically significant reduction of 63%, 61%, 41% and 41% in subepidermal microblister formation, epidermal ulceration, epidermal necrosis and encrustation, respectively. The healing marker, grade of acanthotic area, was elevated by 73% as compared to the pure control. N66 caused also statistically significant reduction in two dermal markers for tissue damage, acute inflammation (33%) and dermal necrosis (48%).

Comparison between control and pure control demonstrates statistically significant reduction in subepidermal microblister formation (49%) and epidermal ulceration (50%) while similar trend of protection, although not statistically significant, was observed with epidermal necrosis, epidermal ulceration and grade of acanthotic area. The same comparison showed statistically significant decrease of 33% in dermal necrosis and similar trend was observed with acute inflammation.

Protective effect of iodine formulation against heat- and HF-induced skin lesions

Previous studies have shown that the topical preparation was not specific against SM, it had beneficial effect against other alkylating agents such as nitrogen mustard (mechlorethamine), iodoacetate, divinylsulfone and cantharidine. Recent studies in our laboratory have demonstrated the protective effect of iodine against other skin irritating factors such as heat stimulus (Fig. 15) and hydrofluoric acid (Fig. 16). For details see the attached paper. It was demonstrated that both prophylactic and post-exposure treatments had beneficial effect against

skin lesions induced by hot water. Hydrofluoric acid-induced skin lesions were also beneficially affected by post exposure treatment with our topical iodine preparation.

SM toxicokinetics in skin

The rate of disappearance of SM from the skin after exposure to 1 μ l (1.27 mg) of the neat irritant is illustrated in Fig. 17. As shown, a relatively fast decrease in skin SM content was observed in the male guinea pig, in particular. In both fur-covered and hairless animals the SM levels measured 60 min after exposure were 0.6% and 0.3% (5.1 and 4.5 μ g), respectively, of the measured, initially applied SM dose. However, a relatively slower kinetics was observed in the female fur-covered guinea pig; an averaged quantity of 521 μ g SM (60.8% of measured initial dose) was determined after 60 min. Only after 180 min post-exposure to the SM, the skin content dropped to as low as 0.4% of the measured initial dose. SM quantities extracted from skin of male fur-covered and hairless guinea pigs immediately after 16 min of exposure to SM vapor were 12.2 and 21.8 μ g, respectively (Fig 18); they declined to 1.6 and 1.7 μ g after 30 and 15 min from termination of exposure, respectively.

Decontamination of SM by hypochlorite *in vivo* and *in vitro*

Topical treatment of SM-exposed skin with 0.5% hypochlorite (3 successive swabbing with a gauze pad soaked in the decontaminant) caused reduction of 68% in skin SM content (Fig. 19b-H), as compared to the value determined after no swabbing had been performed. Similar findings were obtained when hypochlorite was replaced by water (64% reduction, Fig. 19b-W). The SM content in each of

the three pads was 59, 38 and 25 μg for the first, second and third decontamination processes with water, respectively (Fig. 19a). No SM was detected in the gauze pads soaked with 0.5 % hypochlorite solution. This implies on an effective inactivation reaction by the decontaminant in the pad in contrast to the skin in which it had a negligible effect. *In vitro* studies (Fig. 20) showed that incubation of SM with 0.5% hypochlorite solution at a ratio of 1:10 (v/v) did not cause SM inactivation while 4% hypochlorite reduced SM levels by 17%. However, at a ratio of 1:1000 SM:decontaminant, both 0.5% and 4% hypochlorite reduced SM levels by 92% and 99%, respectively.

Protective effect of N77 against SM skin toxicity in the pig

Preliminary studies with the pig model failed to show protective effect of our formulation (N66) against SM toxicity. We assumed that this failure resulted from the relatively high content of fat in pig skin, resulting in inability of the active components of N66 formulation to penetrate and exert their protective effect. Therefore, we incorporated ethyl alcohol into the formulation in order to convert it more lypophilic and causing better penetration into the skin, resulting in significant protection (Fig. 21).

Effect of mustard on skin collagenolytic activity and the effect of iodine.

Recently we have shown that post exposure treatment with povidone iodine (PI) protects against nitrogen and sulfur mustard-induced skin lesions. Since proteolytic activity is involved in skin damage caused by chemical irritants, we studied the effect of iodine on mechlorethamine (HN2)-induced skin collagenolytic activities in the haired guinea pig model (Fig. 22). The matrix metalloproteinase-9 (MMP-9) activity increased by 30%, 46%, 12% and 23% after 3, 24, 48 and 72 hr of HN2

exposure, respectively, whereas the MMP-2 was elevated by 8%, 65%, 8% and 30%, respectively. Topical treatment with PI 15 and 120 min after HN2 exposure decreased the MMP-9 activity by 67% and 60%, respectively, when skin was analyzed 3 hrs after exposure. The same trend was observed in the MMP-2 and MMP-1 activities after PI treatment. A stronger effect of PI treatment 15 min following exposure was observed in skin analyzed 24 hrs after exposure i.e. a decrease of 83% and 88% in MMP-9 and MMP-2 activities, respectively. Similar findings were observed at interval of 120 min between exposure and PI treatment. Much weaker effect was observed in the MMP-1 activity. Similar trend of PI-induced reduction in the 3 types of collagenase activities was found in skin analyzed 48 and 72 hrs after exposure. Reduced collagenolytic activity may serve as one of the mechanisms by which iodine protects the skin against chemical insult.

Effect of SM on COX deficient mice

Since collagenolytic activity may stem from inflammatory response, we checked whether basic components of inflammation can influence SM skin toxicity. We tested the role of prostaglandins and mainly, COX activities, in SM-induced skin toxicity. Therefore, we investigated the response of COX1- and COX2-deficient mice to SM. WE realized that COX2- (but not COX1) deficient mice had statistically significant lower response to SM than the COX1 deficient and wild type mice (Fig. 23). This indicates that COX2 plays a crucial role in the SM-induced skin response to SM.

Additional use for iodine formulations: antidote against dermal exposure of organophosphates

In chemical warfare attack, identification of the toxic agent might be problematic. It would be beneficial to use antidote **against both vesicating and nerve agents**. Figure 24 demonstrates the lethal effect of paraoxon in mice (topical exposure to 4 and 5mg/kg, the upper and lower graphs, respectively), whereas dermal application of iodine formulation 15 min after intoxication kept the animals alive without toxicity signs.

Molecular mechanism of iodine-induced protection against SM

The experimental approach

As mentioned, our primary explanation for the beneficial action iodine was chemical inactivation of SM. We demonstrated that iodine protected the skin against the nitrogen mustard mechlorethamine, **but also against non-oxidizable skin irritants** such as iodoacetate, cantharidine, divinylsulfone (10,11,12) and hydrofluoric acid (37). Furthermore, GC/MS analysis showed that **iodine did not oxidize sulfur mustard** (18). In addition, **our iodine formulation was shown to be efficacious as post-exposure treatment against heat burns** (11,17, 38) and Ultra Violet B (UVB)-induced skin lesions (17). Therefore, we hypothesized that **iodine-induced protection stems from a pharmacological/biological intrinsic action of iodine on the skin and not from SM oxidation.**

Our recent Studies

Indications for iodine-induced skin protective factors

We assumed that iodine might operate by **induction of intrinsic protective factor(s)** responsible for defending the tissue against the noxious stimulus. In order to confirm this

hypothesis, guinea pigs were exposed to 75°C water for 10 sec, immediately thereafter iodine was applied for 2 hrs. The treated skin (13.7 cm²) was extracted with 40 ml ethanol for 2 hrs. The extract was concentrated by evaporation, diluted with saline and injected intradermally into naive guinea pigs.

As shown in Fig. 25 the heat-induced ulceration area in the extract-injected animals was significantly lower by 68% than that of the control animals. *These findings indicate for the induction of protective factor(s) in the iodine-treated skin burns.*

Chemical identification of the protective factors

HPLC fractionation combined with HPLC/MS/MS and sequence analysis identified 4 main peptides of two groups:

H-Lys¹-Gly²-Asn³-Tyr⁴-Ala⁵-Glu⁶-Arg⁷-Ileu⁸-Ala⁹-OH (peptide III)

H-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg-OH (peptide IV)

H-Thr-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg-OH (peptide VI)

H-Thr-Thr-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg-OH (peptide VII)

Peptide III is a partial sequence of Histone H2A whose carboxy terminal Gly⁹ was substituted by Ala. Peptide VI was identified as guinea pig fibrinopeptide A while peptides IV and VII differ from peptide VI by omission and addition, respectively, of an amino terminal threonine moiety.

Counter-irritating activity of the peptides

We have synthesized the peptides and tested their antidotal activity against chemical and thermal stimuli. The peptides were intradermally injected 5 min prior exposure to SM or heat. In order to prevent peptide degradation a cocktail of proteinase inhibitors was administered with the peptides. The peptides/proteinase inhibitors mixture caused statistically significant reduction of 76% and 45% in ulceration area caused by thermal and chemical (SM) stimuli,

respectively (Fig. 26).

We have also tested the counter-irritating activity of each peptide separately against chemical (SM-induced) skin burns. Peptides III and IV were the most potent antidotes against SM; they reduced ulceration area by 67% and 62%, respectively (Fig. 27).

Development of metabolically stable analogs

In order to stabilize peptide against proteolytic activity, we employed N-methylation of the amide bond in which the hydrogen atom of the nitrogen (R-CO-NH-R) was substituted by a methyl group (R-CO-NCH₃-R). This type of modification is not recognized by proteolytic enzymes (39). We have successfully developed biologically active, metabolically stable N-methylated analogs of the neuropeptide substance P (39,40) and employed this experience in the current study(40).

We synthesized a series of N-methylated analogs of peptide III and tested their protective activity against SM-induced skin toxicity (shown in Table 1). The analogs were prepared at the Interdepartmental Unit, Givat Ram, The Hebrew University. The synthesis was carried out by peptide synthesizer using the appropriate F-moc-N-methyl amino acids and appropriate reagents. The peptides were purified by HPLC using semipreparative Vydac C18 column and analyzed by MS.

Our primary approach was to gain protection against aminopeptidases. Therefore we introduced the N-methyl modification into the N-terminal moiety Gly² (analog 3d, Table 1). Additionally, in an attempt to block certain kinds of endopeptidase activities, we also introduced the N-methyl modification into the Ala⁵ (analog 3b) and Ileu⁸ (3g) moieties. Our experience with N-methylated analogs of Substance P showed that N-methylation may confer protection on adjacent peptide bonds (40) while N-methylations of two

peptide bonds may produce highly resistant analogs against proteolytic activity (40).

Therefore we prepared derivatives containing di- (analog 3e) and tri- (analog 3h) N-methylations.

Table 1: Structural formula and biological activity of N-methylated analogs of peptide III.

<u>#</u>	<u>molecular structure</u>	<u>% protection</u>
3b	H-Lys ¹ -Gly ² -Asn ³ -Tyr ⁴ -MeAla ⁵ -Glu ⁶ -Arg ⁷ -Ileu ⁸ -Ala ⁹ -OH	81**
3g	H-Lys ¹ -Gly ² -Asn ³ -Tyr ⁴ -Ala ⁵ -Glu ⁶ -Arg ⁷ -MeIleu ⁸ -Ala ⁹ -OH	72*
3d	H-Lys ¹ -MeGly ² -Asn ³ -Tyr ⁴ -Ala ⁵ -Glu ⁶ -Arg ⁷ -Ileu ⁸ -Ala ⁹ -OH	29
3e	H-Lys ¹ -MeGly ² -Asn ³ -Tyr ⁴ -Ala ⁵ -Glu ⁶ -Arg ⁷ -MeIleu ⁸ -Ala ⁹ -OH	31
3h	H-Lys ¹ -MeGly ² -Asn ³ -Tyr ⁴ -MeAla ⁵ -Glu ⁶ -Arg ⁷ -MeIleu ⁸ -Ala ⁹ -OH	0

Me means N-methyl derivatization. Percent protection means % of reduction in ulceration area in the peptide-treated group in comparison to that of the control (vehicle) group (practically we subtracted the ulceration area of the peptide-treated group from that of the control (vehicle) group and calculated the % of obtained value from the control area). * and ** indicate statistically significant difference (Mann-Whitney U test) from control $p < 0.001$ and 0.0005, respectively. The protective effect of the N-methylated peptides is illustrated in Fig. 28.

A patent application was filed on these inventions (41).

Testing the protective peptides in the mouse ear swelling test

The peptides were also evaluated in the mouse ear swelling test. We realized that there was a protective effect of iv injection of peptides IV and 3b against the inflammatory response caused by sulfur mustard (SM). Peptide IV and 3b at a dose of 40mg/kg reduced the SM-induced inflammatory response by 19% and 18%, respectively (Fig. 29). Lower doses of 10mg/kg were ineffective.

H-Lys¹-Gly²-Asn³-Tyr⁴-**Me**Ala⁵-Glu⁶-Arg⁷-Ileu⁸-Ala⁹-OH (peptide 3b)

H-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg-OH (peptide IV)

Our interpretation for the relatively low efficacy of the peptides in the mouse ear model was that the peptides are species specific, namely, each species is affected by its own sequence. Therefore, we synthesized mouse and human histone H2A 36-44 sequences and tested them in combination with antibodies to the main proinflammatory cytokines, tumor necrosis factor (TNF α) and interleukin 1 (IL1 beta).

The ability of the novel peptides of the invention to enhance the activity of known anti-inflammatory agents was tested using the novel peptides in conjunction with modulatory antibodies capable of neutralizing cytokines, which are inflammatory mediators

Fig. 30 demonstrates the protective effect of the combinations of anti mouse IL1 beta antibodies, anti mouse TNF alpha antibodies, peptide IV and peptide 3m1 against sulfur mustard -induced inflammatory response as expressed by mouse ear edema. This combination reduced SM-induced ear edema by 31%. Omission of peptide IV from this combination reduced mouse swelling by 15%. Peptide IV only has no protective effect. Thus, peptide IV had a synergistic effect on the protection of the combination of anti mouse IL1 beta antibodies, anti mouse TNF alpha antibodies, and peptide 3m1.

H-Lys-Gly-Asn-Tyr-Ser-Glu-Arg-Val-Gly-OH (peptide 3m, mouse rat and human sequence)

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (peptide 3m1, human and mouse sequence)

H-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg-OH (peptide IV)

In the near future we will test additional human H2A histone 36-44 sequences:

H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Val-Gly-OH (Human H2A)

H-Lys-Ala-His-Tyr-Ser-Glu-Arg-Val-Gly-OH (human H2A)

H-Lys-Ser-Arg-Thr-Thr-Ser-His-Gly-Arg-Val-Gly-OH (Human H2A)

We will also synthesize their N-methyl analogs in order to obtain metabolically stable analogs.

We are also planning to test human fibrinopeptide A:

H-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-OH (Human Fibrinopeptide A)

in combination with the H2A fragment and interleukin antibodies.

Conclusions: N-methylation at Ala⁵ (3b) and Ileu⁸ (3g) produced biologically active analogs with significant protective effect. However, substitution of Gly² by N-methyl Gly resulted in significant reduction in the protective activity of all tested analogs. In the mouse ear swelling test the 3b analog had protective activity at a high dose. Treatment of mice with mouse sequence peptide (3m1) combined with fibrinopeptide A and anti TNF and IL1 resulted in significant degree of protection.

PCT application (41) was filed on these findings.

Discussion

The present study further established the protective effect of iodine combined with NSAID and anti-inflammatory steroid against SM in the haired guinea pig and mouse ear models. The toxicokinetic properties of neat and vapor SM after dermal exposure to SM and its inactivation by hypochlorite were also studied. In addition, we demonstrated the effect of our formulation against heat- and hydrofluoric acid-induced skin burns. The iodine-induced protective factors were identified and their N-methylated analogs were synthesized and tested for their protective activity against SM-induced skin toxicity. We found that two of the newly synthesized peptides were pharmacologically active. We also found that the protection by the H2A peptides is species specific, thus each species requires its own sequence in order to be protected. Future studies may lead to a "whole body protection" of the

skin and other sensitive organs such as lung, eye and bone marrow. Moreover, these newly developed peptides may serve as **potent anti-inflammatory agents** for treatment of various inflammation-associated diseases.

KEY RESEARCH ACCOMPLISHMENTS

The present findings address the following modified SOW missions:

- Further establishment of the protective activity of the iodine preparation against neat SM in the haired guinea pig model and in the mouse ear swelling test.
- Verifying the effect of oxidizing agent on SM.
- Identification of iodine-induced protective factors.
- Development of pharmacologically active N-methylated analogs of the protective factor.
- Testing the peptides ip route of administration in the haired guinea pig skin.
- Testing different doses of the peptides by iv route of administration in mouse ear swelling test.

REPORTABLE OUTCOMES

Abstracts and Accompanied Presentations

1. Wormser, U. Brodsky, B. Sintov, A. and Nyska, A. Topical treatment with iodine protects against sulfur mustard-induced skin irritation. 2000, Jerusalem, Israel. P29 (lecture).

2. Wormser, U. Brodsky, B. Sintov, A. and Nyska, A. Iodine protects skin against mustard gas. III Chemical and Biological Medical treatment. 2000, Spiez, Switzerland. P37 (lecture).
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Patents

1. Sintov A. and Wormser, U. compositions containing molecular iodine. Israeli Patent Application No. 135,222 (2000); International Application No. PCT/ IL 01/00277 (2001).
2. Wormser, U. Protective factors against inflammation, burns and noxious stimuli. Israeli Patent Application (2001). PCT submitted 8/2002.

CONCLUSIONS

1. Povidone-iodine together with NSAID and steroidal anti-inflammatory agent protects the haired guinea pig skin against neat SM at intervals of 45 min and less between exposure and treatment.
2. Povidone-iodine together with NSAID and steroidal anti-inflammatory agent protect the mouse ear against SM at intervals of 60 min and less between exposure and treatment.
3. Iodine protects the guinea pig skin against heat- and hydrofluoric acid-induced skin burns.
4. SM is chemically inactivated *in vitro* but not *in situ* by sodium hypochlorite.
5. Mustard induces skin collagenolytic activity; topical treatment with iodine reduces this activity.
6. COX2-deficient mice are less reactive to SN than the COX1 –deficient mice or wild type mice.
7. Iodine formulation protects against dermal exposure of the organophosphate paraoxon.
8. Iodine-induced factors (peptides) protect against SM.

9. The peptides are identified and possess protective activity against SM-induced skin lesions.
10. N-methylated analogs of one of the protective peptides are pharmacologically active against burns caused by SM.
11. Administration of a combination of peptides and proinflammatory cytokine antibodies protects against SM-induced skin toxicity and possesses significant anti-inflammatory action.

“So what”: The present results establish the protective effect of a combination of iodine , piroxicam and clobetasol and offer an efficient topical preparation for post exposure treatment in case of SM exposure of soldiers and civilians. In addition, the newly identified protective peptide and their derivatives and analogs may serve as a) potential prophylactic and/or post exposure treatments against chemical and thermal, b) useful tool for investigating the molecular mechanism of SM activity and of iodine-induced protection, c) anti-inflammatory system for inflammation-associated diseases.

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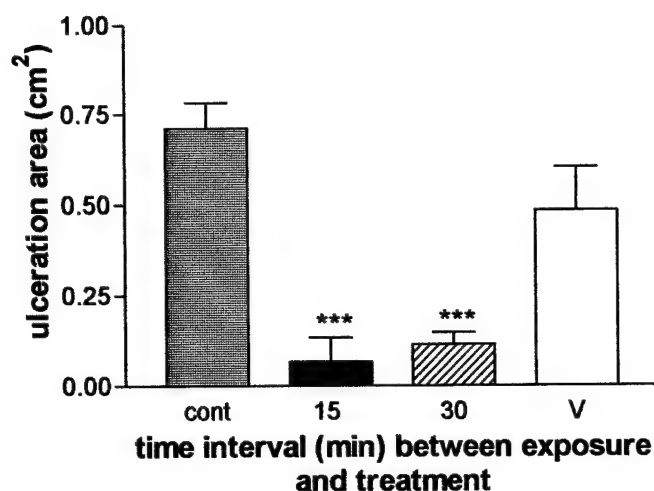
APPENDICES

Fig. 1: Macroscopic appearance of the protective effect of iodine against SM-induced skin lesions.

Six sites of a shaved guinea pig back were exposed to 1.2 mg (1 μ l) neat SM. The 3 left-side sites were treated topically with iodine formulation A 30 min after exposure. Photograph was taken 2 days after treatment.



FIG. 2: Gross pathology quantification of the protective effect of iodine.

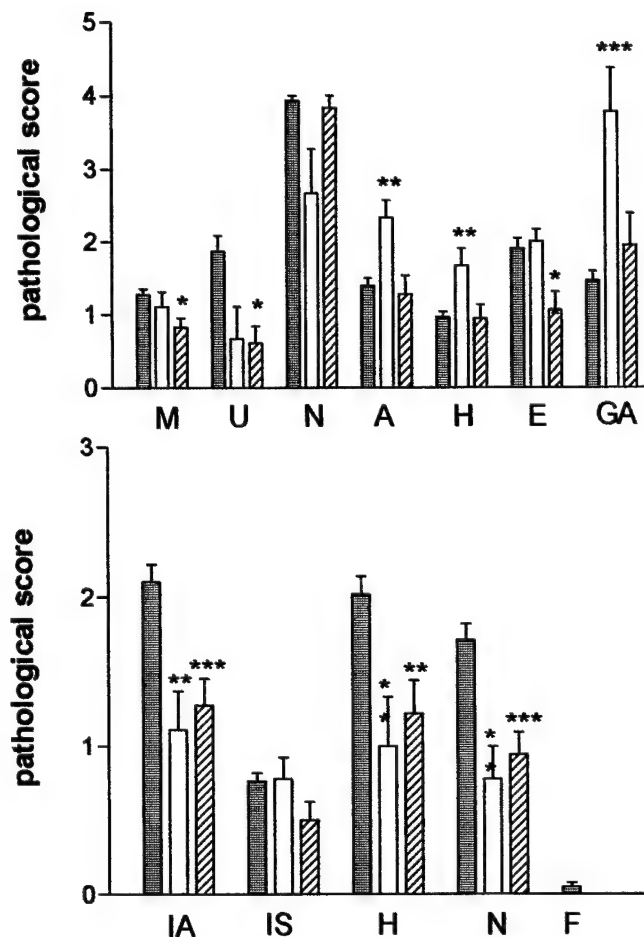


Guinea pigs were exposed to 1.2 mg SM (1 μ l) and were treated topically with iodine formulation A. The ulcerated area was measured 2 days following exposure. The time intervals between exposure and treatment were 15 (n=9) and 30 (n=18) min as indicated. Cont indicates control, SM-exposed skin without iodine treatment (n=57), and V represents vehicle A applied 15 min after SM exposure (n=9). Results are expressed as mean \pm SE using the Kruskal-Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the differences between the experimental groups.

*** $p < 0.001$ at comparison between 15 and 30 min interval and cont.

$p < 0.05$ at comparison between 15 min interval and vehicle.

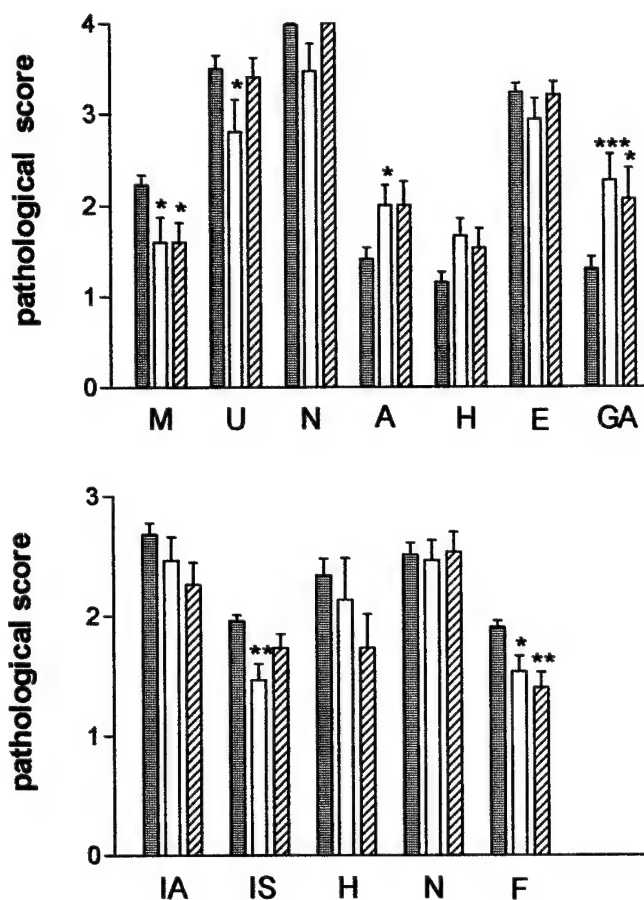
Fig. 3: Histopathology quantification of the protective effect of iodine at intervals of 15 and 30 min between exposure and treatment.



Skin sections (stained with H&E) from guinea pigs exposed to 1.2 mg SM (1 μ l), treated topically with iodine (formulation A) and sacrificed 2 days after treatment. Twelve parameters were determined 7 epidermal (upper part) and 5 dermal (lower part). The epidermal markers were subepidermal microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), and grade of area of epidermal acanthosis (GA). The dermal markers were acute inflammation (IA), subacute inflammation (IS), hemorrhage (H), necrosis (N), and fibrosis (F). The following types of treatment were evaluated: skin exposed to SM only (dotted bars, n=57), skin exposed to SM followed by iodine treatment 15 min (open bars, n=9) or 30 min (hatched bars, n=18) later. Results are expressed as mean \pm SE using Kruskal Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the differences between controls (SM only) and 15 or 30 min interval between exposure and treatment.

*p<0.05; **p<0.01; ***p<0.001

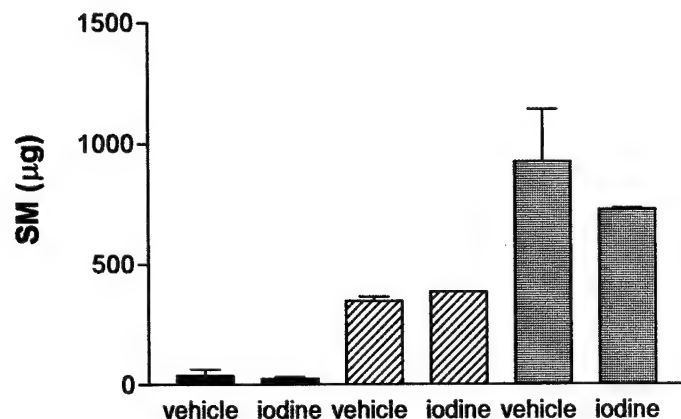
Fig. 4: Histopathologic quantification of the protective effect of iodine at intervals of 45 and 60 min between exposure and treatment.



Skin sections (stained with H&E) from guinea pigs exposed to 1.2 mg SM (1 μ l), treated topically with iodine (formulation A) and sacrificed 6 days after treatment. Twelve parameters were determined 7 epidermal (upper part) and 5 dermal (lower part). The epidermal markers were subepidermal microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), and grade of area of epidermal acanthosis (GA). The dermal markers were acute inflammation (IA), subacute inflammation (IS), hemorrhage (H), necrosis (N), and fibrosis (F). The following types of treatment were evaluated: skin exposed to SM only (dotted bars, n=53), skin exposed to SM followed by iodine treatment 45 min (open bars, n=15) or 60 min (hatched bars, n=15) later. Results are expressed as mean \pm SE using Kruskal Wallis test and Dunnett's multiple comparison post test for statistical evaluation of the differences between controls (SM only) and 45 or 60 min interval between exposure and treatment.

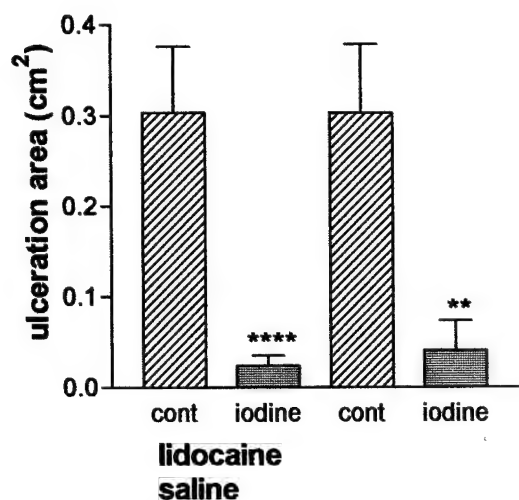
*p<0.05; **p<0.01; ***p<0.001

Fig. 5: Effect of iodine on SM inactivation *in situ*.



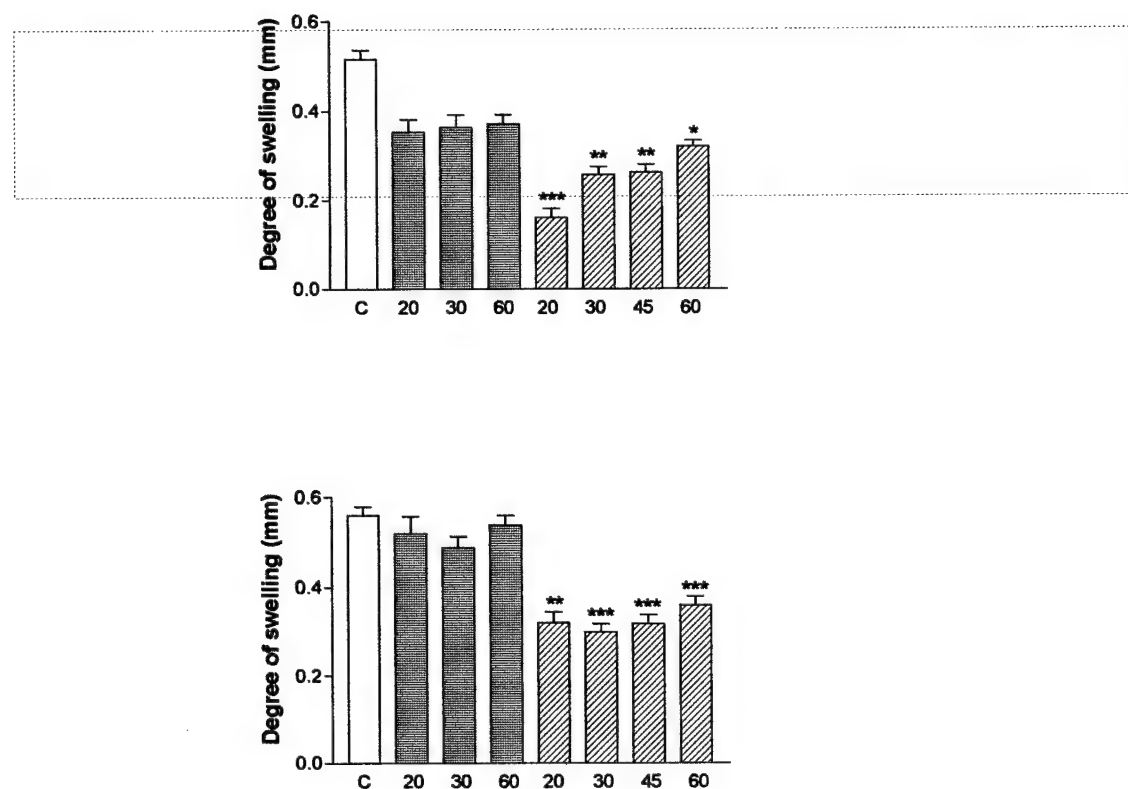
Iodine formulation A or vehicle A were applied 10 min following SM (1µl neat liquid). Twenty min later the iodine solution and the skin were extracted for SM analysis with dichloromethane. Low amounts of SM were detected in the skin (closed bars) after treatment with iodine formulation A or vehicle A. No SM could be detected in the iodine solution or its vehicle (not shown). An additional procedure included application of 1 ml iodine formulation B or vehicle B 20 min following SM exposure. Two h later the iodine or vehicle solution (hatched bars) and the skin (dotted bars) treated with iodine or its vehicle, were extracted with dichloromethane for SM analysis. Results are the mean of 3 experiments.

FIG. 6: Effect of lidocaine on the protective effect of iodine.



The skin area was locally anesthetized with lidocaine (2%, 0.1 ml) injected sc about 1 cm laterally to each site of exposure so that each animal received 6 injections. For comparison, control animals were injected with saline. Twenty min later, six sites on each animal back were exposed to 1.2 mg neat SM (marked as cont, hatched bars). Iodine formulation C ointment was topically applied after 15 min (dotted bars). Lidocaine or saline treatment was repeated 2 h after the first injections. Ulcerated areas were measured 7 days after exposure. Results are expressed as mean \pm SE using the Mann-Whitney two-tailed test for statistical evaluation of the differences between: a) the control (SM only, n=21) and iodine treatment (n=21) of the lidocaine-injected animals (**** $p < 0.0001$), b) the control (n=18) and iodine treatment (n=15) of the saline-injected animals (** $p < 0.01$).

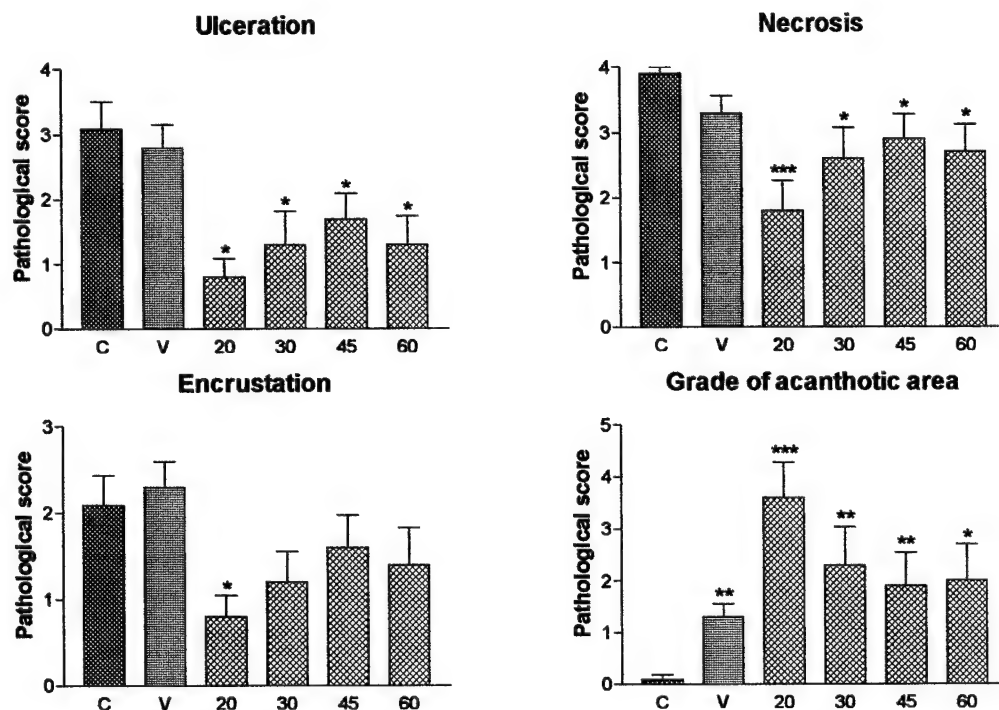
Fig 7: Effect of N66 and its vehicle against SM-induced mouse swelling.



Mouse ear thickness was measured 24 (upper graph) and 48 (lower graph) hours after SM (318 μ g) exposure and subtracted from that measured before exposure. Numbers in the X axis indicate time intervals (min) between exposure and treatment with the vehicle (dotted bars) and N66 (hatched bars). C indicates SM exposure without further treatment (open bars). Results are expressed as mean \pm SEM using Mann-Whitney U test for statistical evaluation of the difference between the effect of N66 treatment and its vehicle for each time interval between exposure and treatment. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0001$.

Fig. 9: Histopathology quantification of the protective effect of N66 in the epidermis of mouse ear.

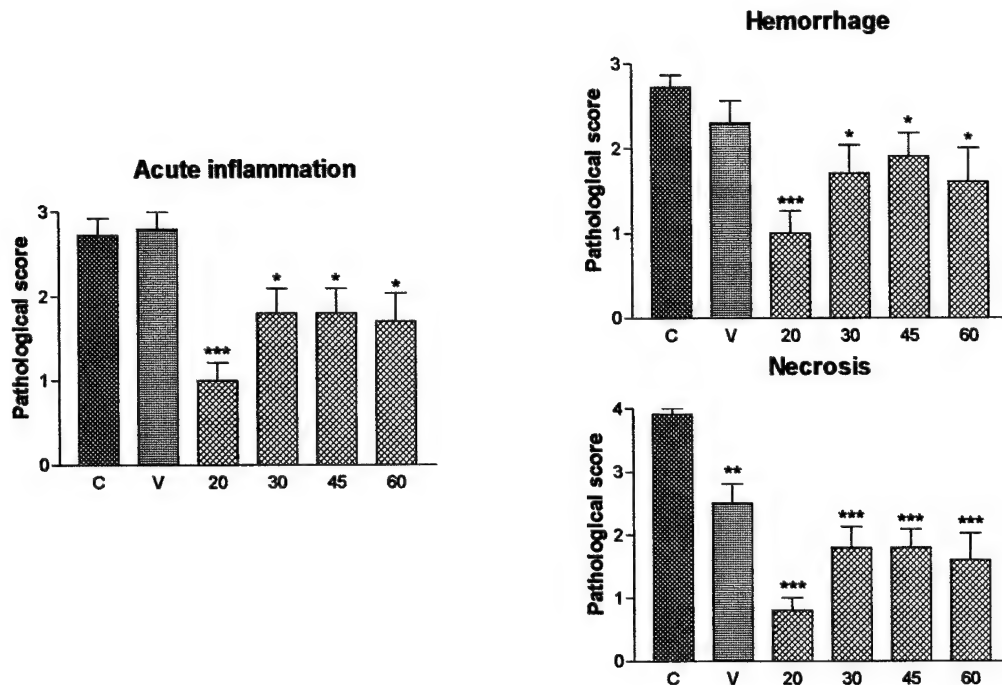
Epidermal parameters



Ear sections (stained with H&E) from mouse exposed to 318 μ g SM and treated topically with N66 preparation 20, 30, 45 and 60 min after exposure. The animals were sacrificed 2 days after treatment. Four epidermal parameters are shown, ulceration, necrosis, encrustation and grade of acanthotic area. The following types of treatment were evaluated: ear exposed to SM only (C; n=11), skin exposed to SM and treated with N66 after 20, 30, 45 and 60 min (n=10 for each time interval). The vehicle (V) was applied 30 min following exposure. Results are expressed as mean \pm SEM using the Mann-Whitney U test for statistical evaluation.

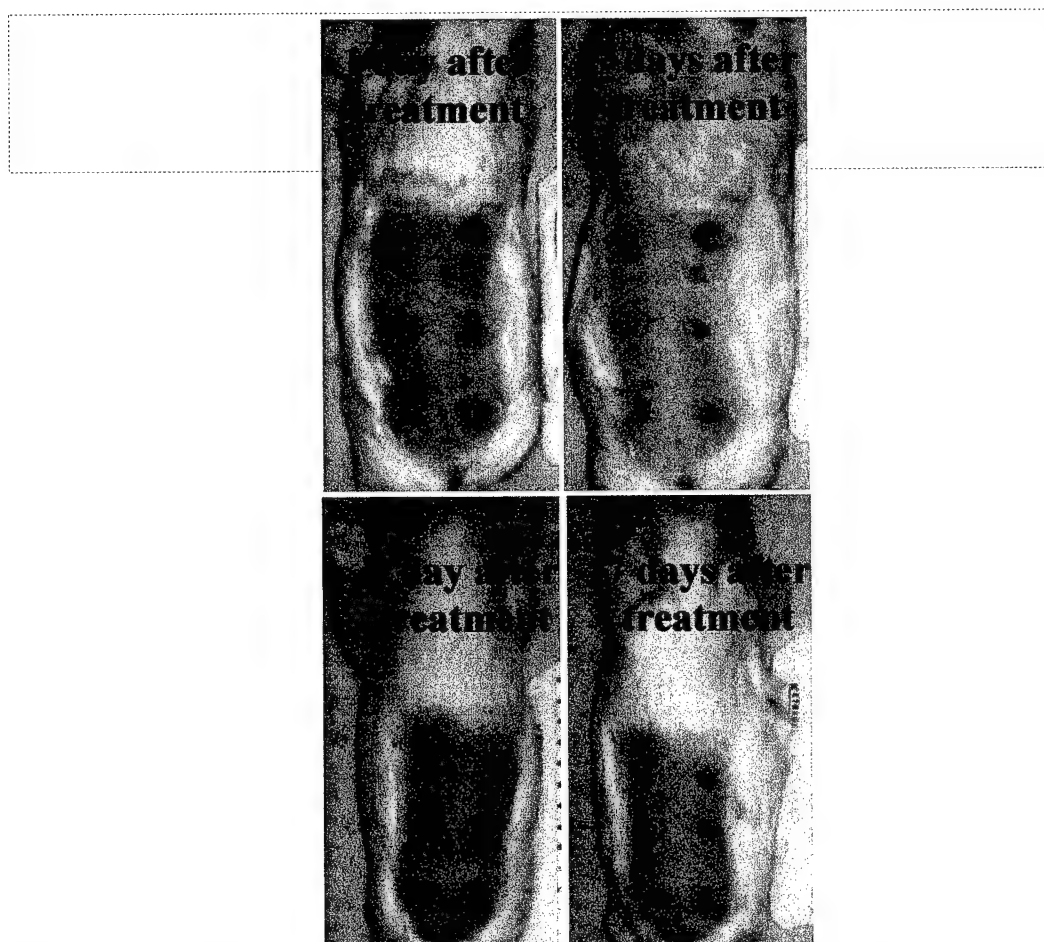
Fig. 10: Histopathology quantification of the protective effect of N66 in the dermis of mouse ear.

Dermal parameters



Ear sections (stained with H&E) from mouse exposed to 318 μ g SM and treated topically with N66 preparation 20, 30, 45 and 60 min after exposure. The animals were sacrificed 2 days after treatment. Three dermal parameters are shown, acute inflammation, hemorrhage and necrosis. The following types of treatment were evaluated: ear exposed to SM only (C; n=11), skin exposed to SM and treated with N66 after 20, 30, 45 and 60 min (n=10 for each time interval). The vehicle (V) was applied 30 min following exposure. Results are expressed as mean \pm SEM using Mann-Whitney U test for statistical evaluation of the difference between the effect of N66 treatment and the control (SM only) for each time interval between exposure and treatment. * p <0.05; ** p <0.01; *** p <0.001

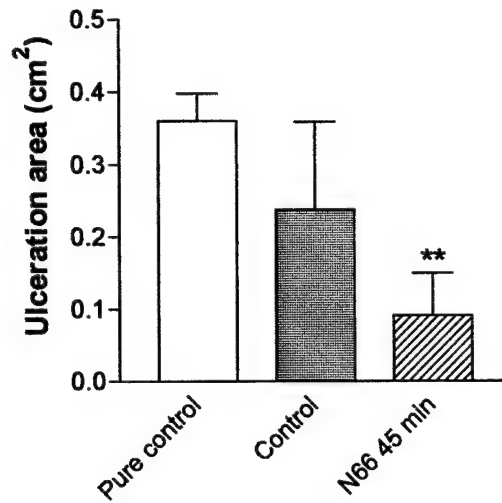
Fig 11: Macroscopic appearance of the protective effect of N66 and its vehicle against SM-induced skin lesions.



Upper photos: Six sites on a shaved guinea pig back were exposed to 1.27 mg (1 μ l) neat SM. The three left sites were topically applied with N66 45 min after exposure. The right sites did not receive treatment (C, control).

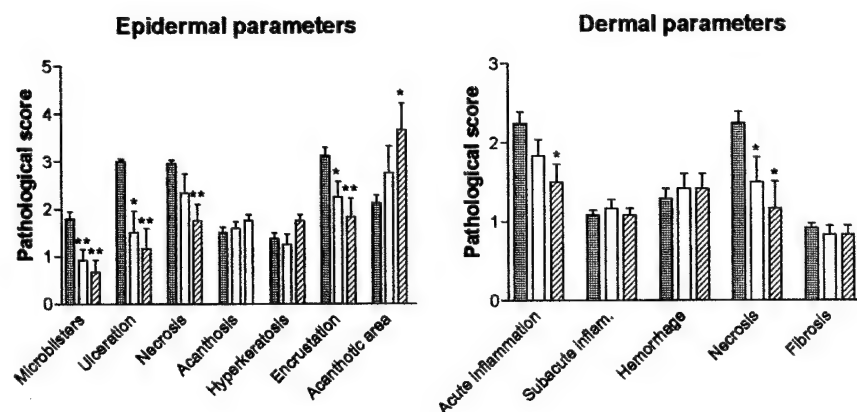
Lower photos: Six sites on a shaved guinea pig back were exposed to 1.27 mg (1 μ l) neat SM. The right left sites were topically applied with vehicle 45 min after exposure. The left sites did not receive treatment (C, control). Photos were taken 1 (left) and 7 (right) days after exposure.

Fig. 12: Gross pathology quantification of the protective effect of N66 in the guinea pig.



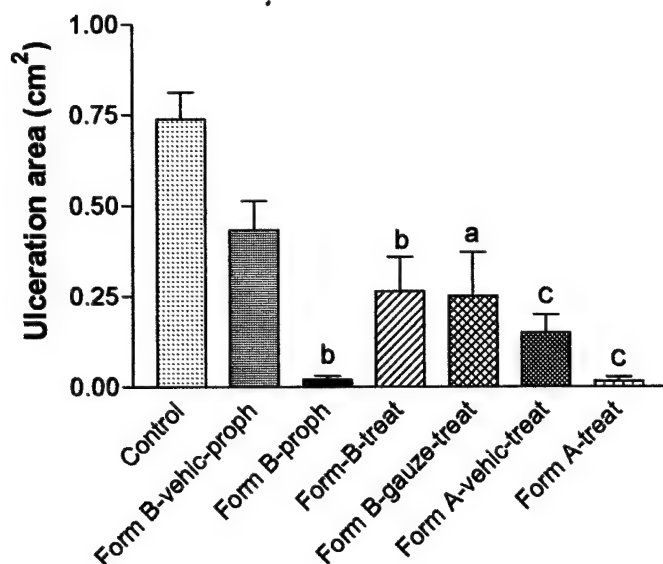
Shaved backs of animals were exposed to 1.27 mg SM (1 μ l) and treated topically with N66 or its vehicle 45 min after exposure. Control means guinea pig skin that was exposed to SM only but adjacent sites were treated with iodine 45 min after exposure. Pure control means skin exposed to SM only and the entire skin surface was not treated with N66. The ulcerated area was measured 7 days following treatment. Results are expressed as mean \pm SEM using Mann-Whitney U test for statistical evaluation of the difference between the effect of N66 treatment, the control and pure control. * $p < 0.01$ for comparison between N66 45 min and pure control.

Fig. 14: Histopathologic quantification of the protective effect of N66 against SM in the guinea pig skin model.



Skin sections (stained with H&E) from guinea pigs exposed to 1.27 mg SM (1 μ l), treated topically with N66 and sacrificed 7 days after treatment. Twelve parameters were determined 7 epidermal (left part) and 5 dermal (right part). The epidermal markers were subepidermal microvesicle formation, ulceration, necrosis, acanthosis, hyperkeratosis, encrustation and grade of area of epidermal acanthosis (acanthotic area). The dermal markers were acute inflammation, subacute inflammation, hemorrhage, necrosis, and fibrosis. The following types of treatment were evaluated: skin exposed to SM only taken from animals that did not receive N66 treatment at all (pure control, dotted bars, n=24), skin exposed to SM only but adjacent sites of the back were treated with N66, (control, open bars, n=12), skin treated with N66 45 min following SM exposure (hatched bars, n=12). Results are expressed as mean \pm SEM using Mann-Whitney U test for statistical evaluation of the difference between the pure control and control group, and between the pure control and the N66 treated group. *p<0.05; **p<0.01

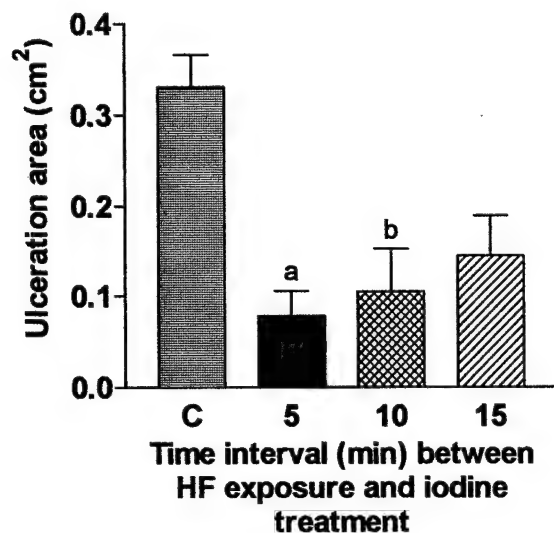
Figure 15. Gross pathological quantification of the protective effect of iodine upon thermal burns.



Each site on guinea pig back was exposed to 75°C water for 10 sec; iodine treatment groups included immediate post-exposure treatment with iodine formulation B (liquid in well, form B-treat, n=18), prophylactic treatment with formulation B (in well, form B-proph, n=9), post-exposure treatment with gauze pad soaked in formulation B (form B-gauze-treat, n=9), post-exposure treatment with iodine formulation A (ointment, form A-treat, n=9), post-exposure treatment with formulation A vehicle (form A-vehic-treat, n=18), prophylactic treatment with formulation B vehicle (form B-vehic-proph, n=18); control group (n=81) did not receive any treatment; ulcerated areas were measured 4d following exposure. Results expressed as mean \pm SEM using Mann-Whitney U-test for statistical evaluation of differences between experimental groups;

a $p < 0.05$, b $p < 0.005$, c $p < 0.001$ —comparison between each experimental group and the control; statistical significance ($p = 0.02$) observed between prophylactic treatment with formulation B and its vehicle.

Figure 16. Gross pathological quantification of the protective effect of iodine upon HF-induced skin lesions.



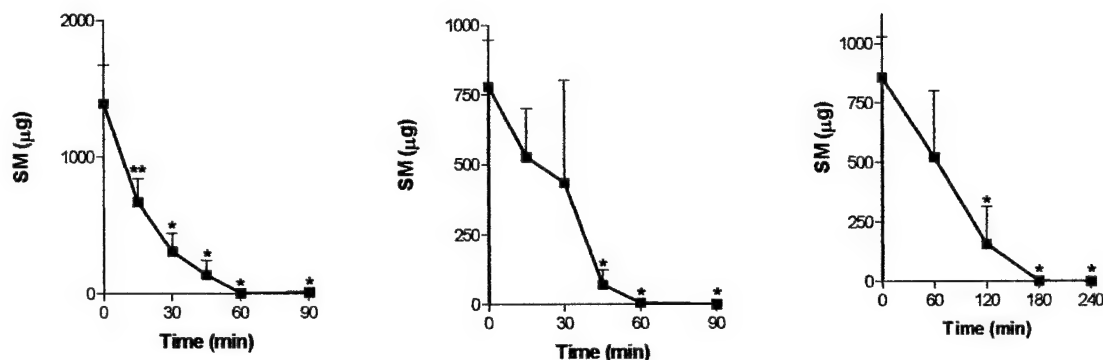
Each site on the guinea pig back was exposed to 10 μ l 60% HF; iodine ointment (formulation A) was applied 5 (n=12), 10 (n=6), and 15 (n=9) min after HF exposure; controls (C, n=27) did not receive iodine treatment; ulcerated areas were measured 3 d following exposure.

Results are expressed as mean \pm SEM using the Kruskal-Wallis test and Dunnett's multiple comparison post-test for statistical evaluation of differences between experimental groups;

a $p < 0.001$ for comparison between 5 min interval and control;

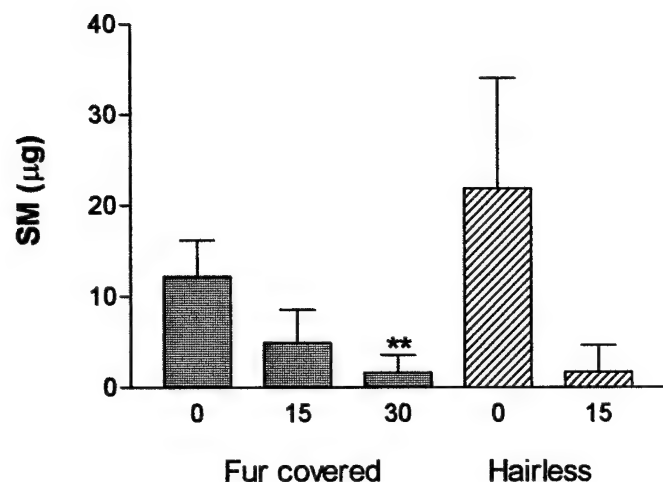
b $p < 0.05$ for comparison between 10 min interval and control.

FIG. 17: Skin toxicokinetics of neat SM



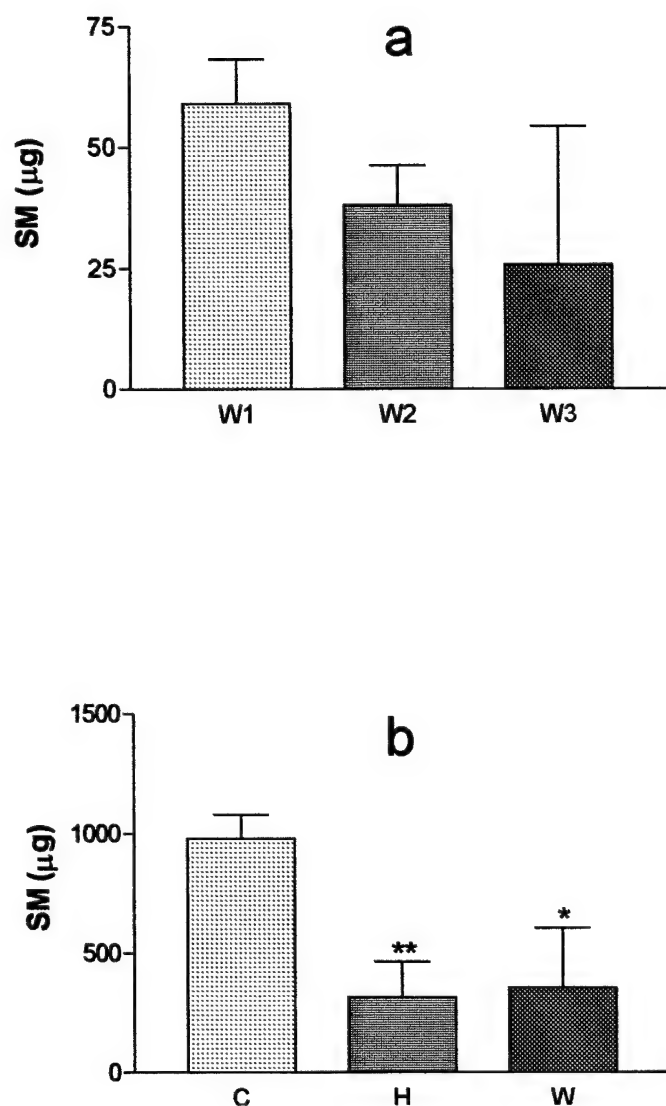
SM (1μl) was applied on the backs of shaved, anesthetized male hairless (left), male fur-covered (middle) and female fur-covered (right) guinea pigs. At the indicated time intervals after exposure, SM was extracted from the skin by dichloromethane and analyzed by GC/MC as described in Materials and Methods section. Each time point is the mean \pm SD of 3 determinations. It is noteworthy that there was no full recovery of SM in the male fur-covered guinea pig skin (779μg instead of 1270μg), which may be due to a rapid reaction with skin components. Statistical significance of the difference between values obtained at each time point from that of zero time was evaluated by student t test. ** $p < 0.05$, * $p < 0.02$

Fig 18: Skin toxicokinetics of SM vapor.



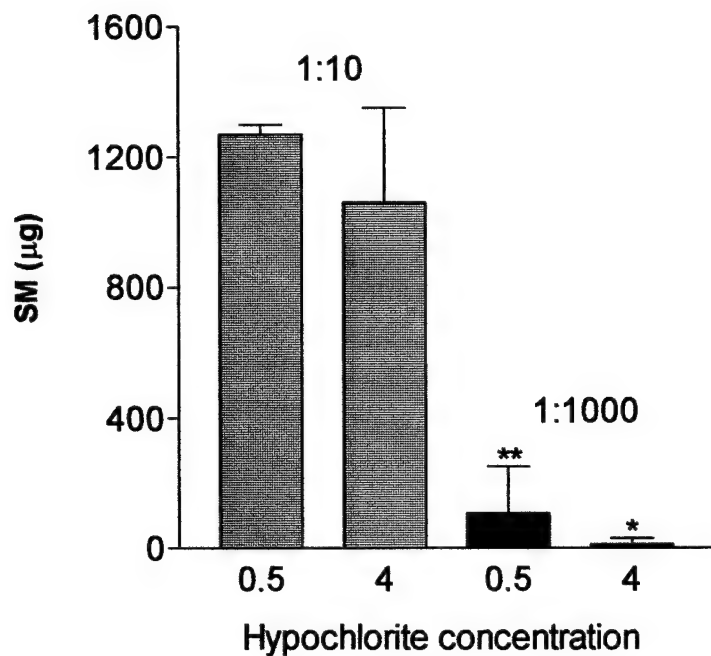
Backs of shaved, anesthetized male fur-covered (dotted bars) and hairless (hatched bars) guinea pigs were exposed to SM vapor for 16 min. At the indicated time intervals after termination of exposure (0 indicates within 1 min), SM was extracted from the skin by dichloromethane and analyzed by GC/MC as described in Materials and Methods. Each time point is the mean \pm SD of 3 determinations. Statistical significance of the difference between value obtained at each time point from that of zero time was evaluated by student t test. ** $p < 0.05$

FIG. 19: In situ skin decontamination of SM by sodium hypochlorite and water.



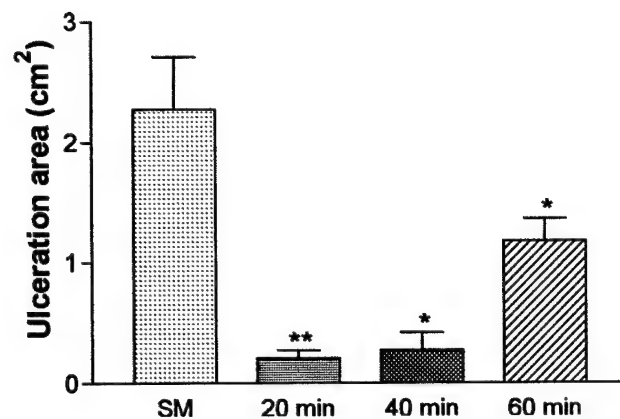
A shaved back of fur-covered guinea pig was exposed to SM (1µl neat liquid). [a] Ten min later the skin was swabbed thrice (W1, W2, W3) with 3 layers of gauze pad soaked in water. The pads were analyzed for total SM content. When water was replaced by 0.5% hypochlorite solution, no SM was detected in the pads. [b] Skin SM was quantified by GC/MS after hypochlorite (H) and water (W) swabbing compared with no swabbing procedure (C). Exposed skin sites were immediately extracted with dichloromethane after swabbing as described in Materials and Methods. Each bar is the mean \pm SD of 3 determinations. Statistical significance of the difference between value obtained with hypochlorite (H) and water (W) treatment, and control (C) was evaluated by student t test. ** $p < 0.01$, * $p < 0.05$

Fig. 20: SM decontamination by sodium hypochlorite in vitro.



SM (1µl neat) was incubated with 10µl (1:10 v/v, dotted bars) or 1ml (1:1000 v/v, closed bars) sodium hypochlorite (0.5% or 4%). After 20 min of incubation at room temperature SM was extracted and analyzed by GC/MS as described in Materials and Methods. Each bar is the mean \pm SD of 3 determinations using the student t test for statistical evaluation of the difference between 1:10 and 1:1000 SM:hypochlorite ratio at hypochlorite concentrations of 0.5% (** $p < 0.005$) and 4% (* $p < 0.05$).

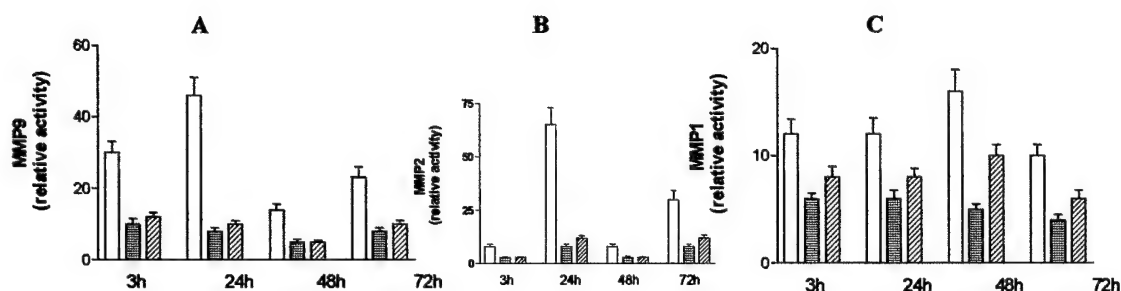
Fig. 21: Protective effect of against SM-induced skin lesions in the pig model.



Anesthetized shaved back of male pig was exposed to SM. Twenty four sites, 12 on each side of the back, were exposed to 1µl neat SM. N77 (povidone-iodine, clobetasol, piroxicam in tetraglycol: ethanol) was applied 20, 40 and 60 min after exposure. Ulceration area was measured after 7 days. Results are expressed as mean±SE using the Mann-Whitney U test for statistical evaluation between the SM exposed and the N77-treated sites.

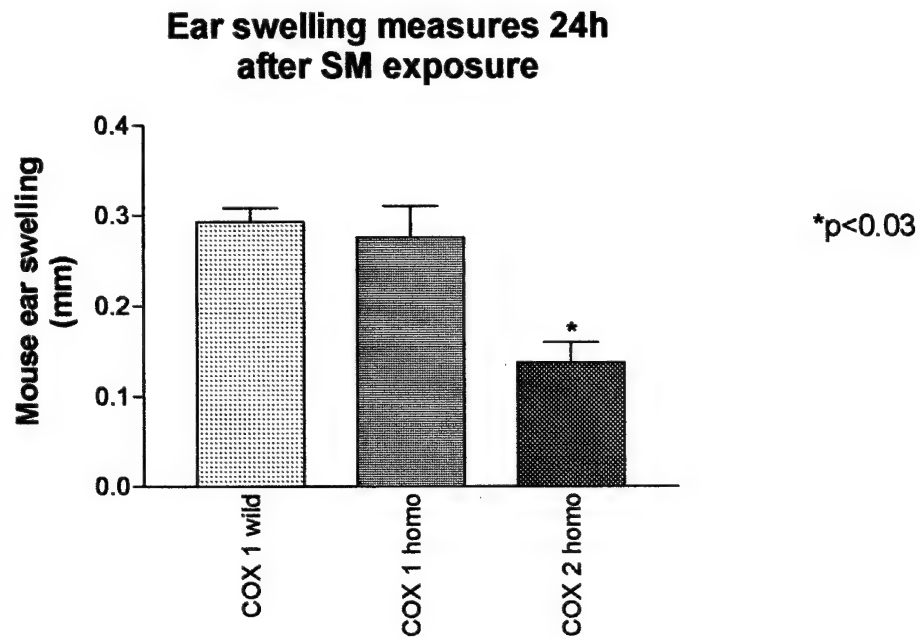
*p<0.05; **p<0.01

Fig. 22: Effect of PI on mechlorethamine-induced skin collagenolytic activity.



Guinea pigs were topically exposed to HN2 (open bars); PI was applied 15 (dotted bars) and 120 (hatched bars) min after exposure. Skin specimens, collected 3, 24, 48 and 96 hrs after PI application, were analyzed for 3 types of collagenolytic activity MMP9 (A), MMP2 (B) and MMP1 (C). Results are expressed as the relative activity between that measured in treated and/or exposed skin and that of control tissue. Each column is the mean \pm SEM of 3 skin samples.

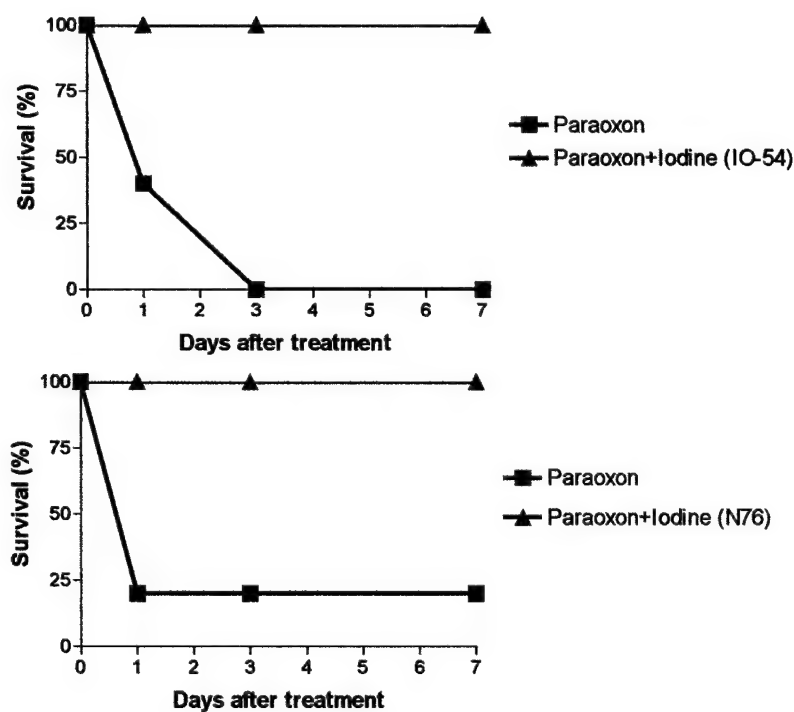
Fig. 23: Effect of SM on COX1- and COX2-deficient and wild type mice



Mice were exposed to SM as described. Ear thickness was measured before and 24 hours after exposure.

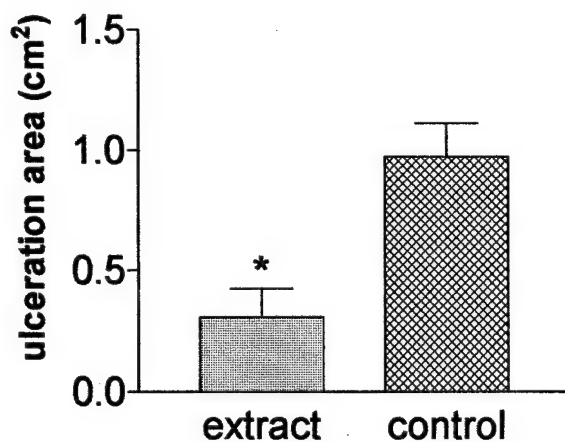
Figure 24: Demonstration of the protective effect of iodine formulation against lethal effect of dermal exposure of paraoxon in mice.

Protective effect of iodine formulations against paraoxon-induced lethality



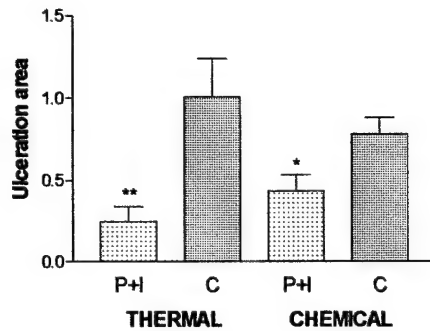
Topical exposure to 4 and 5mg/kg in the upper and lower graphs, respectively, resulted in significant lethal effect whereas dermal application of iodine formulation 15 min after intoxication kept the animals alive without toxicity signs.

Fig. 25: Protective effect of extract of iodine-treated skin against SM-induced skin lesions.



Guinea pigs were exposed to 75°C water for 10 sec, immediately thereafter iodine was applied for 2 hrs. The treated skin (13.7 cm²) was extracted with 40 ml ethanol for 2 hrs. The extract was concentrated by evaporation, diluted with saline and injected intradermally into naive guinea pigs (*p<0.05, Mann-Whitney U test).

Fig. 26: Counter-irritating activity of a combination of all peptides against SM- and hot water-induced skin damage.

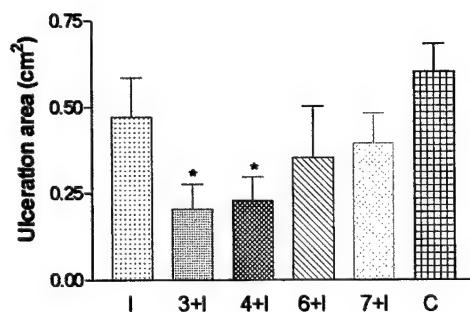


The peptides were intradermally injected 5 min prior exposure to SM or heat. In order to prevent peptide degradation a cocktail of proteinase inhibitors (pepstatin A, chymostatin, antipain, leupeptin) was administered with the peptides (marked as P-I). The control (C) group was injected with the vehicle.

The peptides/proteinase inhibitors mixture caused statistically significant reduction of 76% and 45%

(* $p < 0.05$; ** $p < 0.02$ Mann-Whitney test) in ulceration area caused by thermal and chemical stimuli ($n=12$), respectively.

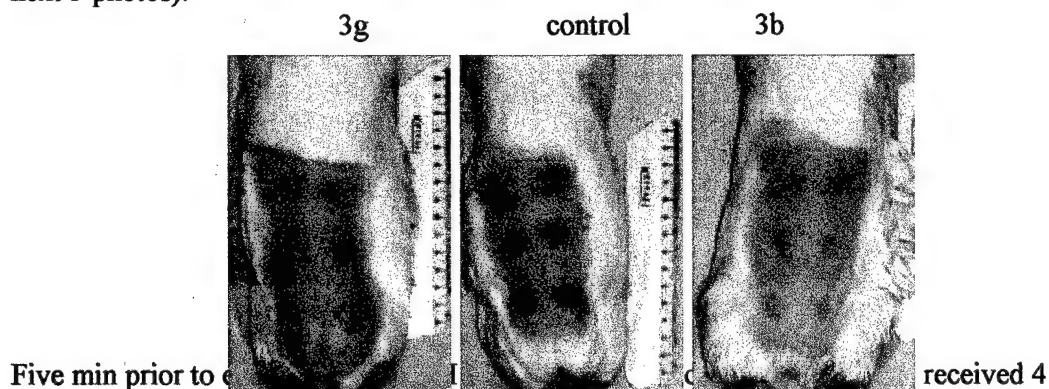
Fig. 27: Testing the counter-irritating activity of each peptide separately against chemical skin burns.



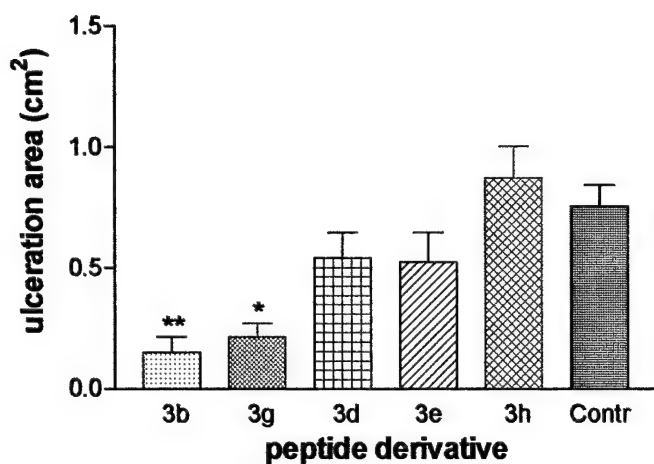
Each peptide is indicated by its arabic number plus I (proteinase inhibitors). I and C indicate proteinase inhibitors only and control, respectively.

Peptides III and IV were the most potent antidotes against SM; they reduced ulceration area by 67% and 62%, respectively (* $p < 0.05$)

Fig. 28: Quantification of the protective activity of the N-methylated analogs against SM-induced skin lesions (the bar graph) and illustration of the effect of peptides 3g and 3b (the next 3 photos).



**Effect of N-methylated analogs
of peptide III on mustard
gas-induced skin burns**

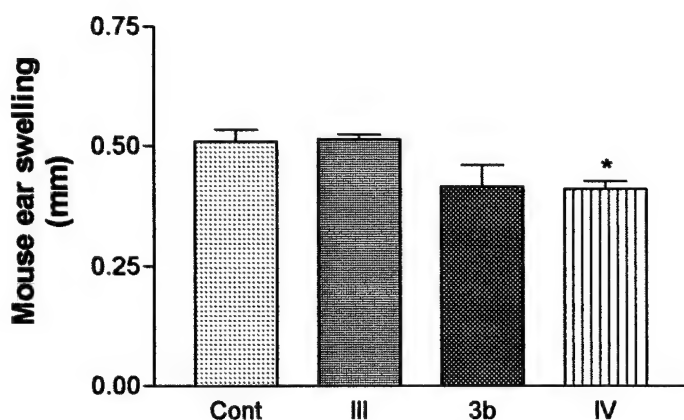


intradermal injections (50 μ l, each at concentration of 5 mg/ml, total volume of 200 μ l per animal) located about 1.5 cm laterally to two adjacent exposure sites. Each analog was separately tested for its ability to protect against SM. Each analog was examined on separate animals. Control (Contr) group was injected with the vehicle (saline, 0.9% NaCl). Ulceration area was measured 3 days after treatment.

- and ** indicate statistically significant (Mann-Whitney U test) from control $p < 0.001$ and 0.0005, respectively

Fig. 29: Protective effect of peptides III, 3b and IV against SM in the mouse ear swelling model.

**Protective effect of peptides III,
3b and IV against SM in the
mouse ear swelling model**



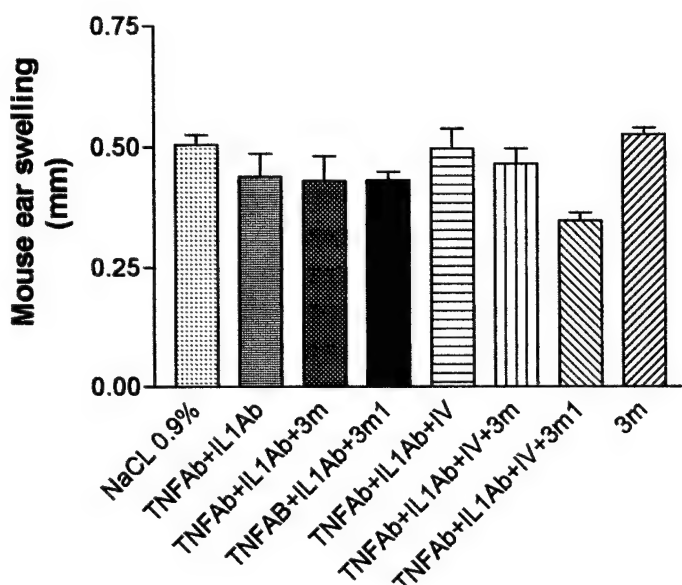
Male ICR mice (~25g) were anesthetized by pentobarbital sodium 60mg/kg ip (0.1ml/25g BW of 1.5% solution) and placed on their abdominal side. Anesthesia was maintained by 0.03ml/25g BW whenever needed. Peptides III, 3b and IV (dissolved in 0.9% NaCl) were injected intravenously (each peptide injected into different group of animals) at a single dose of 40 mg/kg body weight (volume of injection was 0.2 ml). Control (cont) animals received 0.9% NaCl injections. Within 5 min after injection, the outer side of each ear was exposed to 0.317 mg SM (5 μ l of 1:20 dilution in dichloromethane). Mouse ear thickness was measured 48 hours after exposure using micrometer (Model PK-0505, Mitutoyo Corporation, Japan). Edema was assessed by the difference between ear thickness measured after and prior to exposure. Animals were sacrificed 48 hours after exposure. Results are the mean \pm SE using the Mann Whitney U test for statistical evaluation of the difference between Cont and peptide IV group. The asterisk denotes statistical significance at the level of * p <0.01.

H-Lys¹-Gly²-Asn³-Tyr⁴-*Me*Ala⁵-Glu⁶-Arg⁷-Ileu⁸-Ala⁹-OH (peptide 3b)

H-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Val-Arg-OH (peptide IV)

Fig. 30: Synergistic Anti-inflammatory effects of novel peptides with anti-inflammatory agents

Protective effect of anti mouse IL1 β antibodies, anti mouse TNF α antibodies, peptides 3m, 3m1 and IV against SM in the mouse ear swelling model



Male ICR mice (~25g) were anesthetized by pentobarbital sodium 60mg/kg ip (0.1ml/25g BW of 1.5% solution) and placed on their abdominal side. Anesthesia was maintained by 0.03ml/25g BW whenever needed. Peptides 3m, 3m1, IV and IV (dissolved in 0.9% NaCl), and anti mouse IL1beta (IL1Ab) antibodies and anti mouse TNF alpha antibodies (TNFAB) were tested for their ability to protect against SM-induced toxicity. The following combinations were prepared: a) 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies; b) 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg 3m; c) 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg 3m1; d) 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg IV; e) 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg IV, 10mg/kg 3m; f) 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg IV, 10mg/kg 3m1; g) 10mg/kg 3m. All peptides and antibody dilutions were carried out with 0.9% NaCl. Volume of injection was 0.2 ml. Control animals received 0.9% NaCl injections. Within 5 min after injection, the outer side of each ear was exposed to 0.317 mg SM (5 μ l of 1:20 dilution in dichloromethane). Mouse ear thickness was measured 48 hours after exposure using micrometer (Model PK-0505, Mitutoyo Corporation, Japan). Edema was assessed by the difference between ear thickness measured after and prior to exposure. Animals were sacrificed 48 hours after exposure. Results are the mean \pm SE using the Mann Whitney U test

for statistical evaluation of the difference between Cont and peptide IV group. Results are the mean \pm SE using the Mann Whitney U test for statistical evaluation of the difference between 0.9% NaCl group and experimental groups. The asterisk denotes statistical significance at the level of $p < 0.04$.

The same statistical significance was observed for the difference between the group of: 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg 3ml, and the group of: 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg IV, 10mg/kg 3ml;

The same statistical significance was observed for the difference between the group of: 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg IV, and the group of: 5 μ g/injection anti mouse IL1 beta antibodies, 10 μ g/injection anti mouse TNF alpha antibodies, 10mg/kg IV, 10mg/kg 3ml

H-Lys-Gly-Asn-Tyr-Ser-Glu-Arg-Val-Gly-OH (peptide 3m)

H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (peptide 3ml)

H-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg-OH (peptide IV)

Topical Iodine Preparation as Therapy against Sulfur Mustard-Induced Skin Lesions¹

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Sulfur mustard (SM) is a powerful vesicant employed as an agent of chemical warfare. This study demonstrates the therapeutic effect of a novel topical iodine preparation as a postexposure treatment against SM-induced lesions in the fur-covered guinea-pig skin model. Iodine treatment 15 min after SM exposure resulted in statistically significant reductions of 48, 50, and 55% in dermal acute inflammation, hemorrhage, and necrosis, respectively, whereas, the epidermal healing markers, hyperkeratosis and acanthosis, were significantly elevated by 72 and 67%, respectively, 2 days after treatment. At the interval of 30 min between SM exposure and iodine treatment, there was a significant degree of healing or recovery, albeit to a lesser extent than that observed in the shorter interval. Although the epidermal healing markers were not elevated, the parameters indicative of active tissue damage, such as subepidermal microblisters, epidermal ulceration, dermal acute inflammation, hemorrhage, and necrosis, were significantly reduced by 35, 67, 43, 39, and 45%, respectively. At the 45-min interval between exposure and treatment, there was also a certain degree of healing or recovery expressed as significant reductions in dermal subacute inflammation, subepidermal microblister formation, and epidermal ulceration, whereas, acanthosis was statistically elevated, indicating an increased healing potential. At the 60-min interval, iodine was less efficacious; nevertheless, a significant reduction in the incidence of subepidermal microblisters and an expansion of the acanthotic area were observed. Gross ulceration was significantly decreased at intervals of 15 and 30 min between exposure and treatment. The local anesthetic, lidocaine, did not alter the therapeutic effect of iodine. SM was not affected chemically by iodine as measured by gas chromatography-mass spectrometry (GC-MS) analysis. These findings suggest that the iodine preparation functions as an antidote against skin lesions induced by SM. © 2000 Academic Press

Key Words: sulfur mustard; mustard gas; iodine; skin toxicity; dermatotoxicity.

Sulfur mustard (SM), also termed mustard gas, is a potent vesicant which has been employed as a chemical weapon in various conflicts during the 20th century (Mellor *et al.*, 1991; Wormser, 1991). It functions as a powerful alkylator and highly cytotoxic blisterogen in both humans and animals (Dacre and Goldman, 1996; Filipek *et al.*, 1997; Mellor *et al.*, 1991; Mershon *et al.*, 1990; Petrali and Oglesby, 1997; Smith *et al.*, 1995a,b; Wormser, 1991; Zhang and Monteiro, 1997). Skin exposed to SM develops erythema within 30 min to several hours after exposure, followed by edema, vesicle and blister formation, ulceration, necrosis, and desquamation (Smith *et al.*, 1995a,b; Smith and Dunn, 1991; Wormser, 1991). The severity of lesions observed after exposure to SM has emphasized the need for an efficient pharmacological antidote against its vesicating activity. The powerful alkylating activity of SM (Ludlum *et al.*, 1994; Matijasevic *et al.*, 1996; Mol *et al.*, 1993) results from its conversion, in aqueous solution, to the highly electrophilic ethylene episulfonium derivative (Wormser, 1991), which can be neutralized by nucleophilic agents. Some protection against SM can be achieved by glutathione derivatives (Lindsay and Hambrook, 1998; Lindsay *et al.*, 1997), cysteine esters (Wilde and Upshall, 1994), and a cysteine precursor (Gross *et al.*, 1997). Despite having some beneficial effects, these agents were not efficacious enough to be used as antidotes. Additional agents, such as arginine analogs (Sawyer, 1998, 1999; Sawyer and Risk, 2000), a calcium channel blocker (Mazumder *et al.*, 1998), niacinamide (Meier and Johnson, 1992), and its combination with promethazine and indomethacin (Yourick *et al.*, 1995), exhibited weak therapeutic effects as postexposure treatment against SM in experimental animals, although some of these agents were beneficial in *in vitro* and *in vivo* systems, particularly if treated prophylactically.

Iodine and iodophors like povidone iodine are widely used as antiseptic agents. Since their bactericidal effect stems from

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the oxidizing activity of iodine, our primary approach was to employ this characteristic to neutralize chemically SM by oxidation of its sulfur atom to the less active sulfoxide form. Earlier studies from our laboratory showed that postexposure treatment with povidone iodine is effective against SM at an interval of 10 min between exposure and treatment; whereas, at longer intervals the treatment is less effective (Wormser *et al.*, 1997). Our results indicated that the formulation of iodine plays a crucial role in its counterirritating activity. Thus, in the present study we developed an iodine formulation capable of dissolving molecular iodine in an aqueous environment, and we demonstrated the potent therapeutic effect of the new iodine formulation against SM-induced skin lesions. The gross and histopathology of guinea-pig skin at different time intervals between SM exposure and iodine treatment were quantified. Because previous studies have shown that neuronal activity is involved in the evolution of skin damage caused by chemical irritants (Veronesi *et al.*, 1995), we tested the effect of the local anesthetic lidocaine on both skin toxicity of SM and the therapeutic effect of iodine. In addition, the effect of iodine on chemical inactivation of SM was also investigated.

MATERIALS AND METHODS

SM synthesis. SM was prepared (Wormser *et al.*, 1997) by mixing 1 ml of 2,2-thiodiethanol (thiodiglycol; Sigma, St. Louis, MI, distilled before use) with 12 ml concentrated HCl (Merck, Darmstadt, Germany) and bubbling nitrogen for several minutes into the reaction mixture. Then the reaction was maintained at 70–75°C for about 90 min or until a clear, small ball of product appeared in the bottom. After cooling to room temperature, the product was removed and washed 5–6 times with 3–5 ml water to remove traces of HCl. The product was dried with CaCl₂ and kept in a closed tube stored in an hermetically closed beaker. The yield was about 80%. GC/MS analysis showed a purity of more than 99%.

Precautionary steps. The work with SM was carried out according to the precautionary procedures of the Ministry of Labor and Welfare, Section of Labor Inspection, and the Department of Safety, The Hebrew University. All stages of synthesis and animal experiments were performed in a continuously operated fume hood with an air flow of 125 ft/min. Investigators wore lab coats and masks. All contaminated glass and disposables were neutralized in a solution containing 1:1 1N NaOH:ethanol. The animals were kept in the hood until the end of the experiment. After sacrifice, the animals were packed in two thick nylon sacks and burned.

Animals. Male guinea pigs (Dunken Hartley, 600–800 g) were supplied by Harlan Laboratories Breeding Center, Ein Karem, Jerusalem, Israel. Following an acclimation period of 7 days to ensure their suitability for the study, animals were randomly assigned to experimental groups. Test animals were kept within a limited-access rodent facility with environmental conditions set to a temperature of 20 ± 2°C, a humidity of 30–70%, and a 12-h light/12-h dark cycle. Temperature and relative humidity were monitored daily. The animals were housed in filter-covered polypropylene cages with solid bottoms and covered with wood shavings as bedding material. Animals were provided *ad libitum* access to a commercial guinea-pig diet (Kofolk 19520, Netanya, Israel), and drinking water was supplied to each cage via propylene bottles with stainless steel sipper tubes. All procedures, maintenance, and treatment of the animals were in accordance with the principles of humane treatment published by the National Academy of Sciences (U.S. Department of Health, 1996). The Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem approved the protocol.

Iodine formulations. Formulation A: 2% iodine (Merck) dissolved in tetraglycol (TG) to water 1:1 (liquid formulation); Vehicle A: TG:water 1:1; Formulation B: 2% I₂, 2.4% NaI, in 45.6% ethanol, 50% water (liquid formulation); Vehicle B: 50% ethanol, 50% water; Formulation C: 2% iodine, 50% TG, 40% poloxamer 407, 8% water (ointment formulation).

Experimental procedure. Backs of haired guinea pigs were shaved 24 h prior to the experiment. The animals were anesthetized by 30 mg/kg sodium pentobarbital ip. Backs were cleaned with wet, soft white paper and allowed to dry. Six sites on each back, three on each side, were exposed to 1 µl (1.27 mg) SM. One milliliter of iodine formulation A was applied on three exposure sites of each animal, while the other three SM-exposed sites remained untreated. Iodine was applied into wells constructed by cutting a plastic tube cover (inner diameter of 1.7 cm) to form an open-ended cylindrical well. A thin layer of commercial silicon sealing ointment was applied to the edge of the well attached to the back of the animal so that liquid inside did not leak out. The well was exposed to SM, and iodine formulation A was applied 15 and 30 min after SM exposure. In an additional series of experiments, the intervals between exposure and treatment were increased to 45 and 60 min. Unless otherwise indicated, the iodine preparation was left on the skin for 2 h. At the end of the procedure, the liquid iodine was aspirated out and the well removed from the skin. Animals were sacrificed using 100 mg/kg sodium pentobarbital ip. Their backs were photographed alongside a ruler using a Kodak 260 digital camera. The ulceration area of each exposure site was assessed grossly. Skin specimens were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5–6 µm, and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

Effect of lidocaine on the therapeutic effect of iodine. Guinea pigs were shaved and anesthetized with sodium pentobarbital. The tested skin area was anesthetized locally with 0.1 ml of 2% lidocaine injected sc about 1 cm laterally to each site of exposure so that each animal received six injections. For comparison, control animals were injected with saline. Twenty minutes later six sites on each animal's back were exposed to 1.27 mg neat (1 µl) SM. After an additional 15 min, a thin layer of 50 mg of iodine formulation C ointment was applied (well system was not used) on three out of the six exposure sites of each animal while the other three exposure sites of the animal remained untreated. Lidocaine or saline treatment was repeated 2 h after the first injections. The effect of lidocaine was proven by the lack of response to mechanical stimulation along the experiment. The ulcerated area was measured 7 days after exposure. Results are expressed as mean ± SE using the Mann-Whitney two-tailed test for statistical evaluation of the differences between the control (SM only) and iodine treatment of the lidocaine-injected animals and the control and iodine treatment of the saline-injected animals.

Determination of the effect of iodine on chemical inactivation of SM *in situ*. Wells were constructed on the backs of pentobarbital-anesthetized guinea pigs as described previously. SM (1 µl neat liquid) was applied in the center of each well followed 10 min later by the addition of 1 ml of iodine formulation A or vehicle A. After an additional 20 min, the entire iodine solution was aspirated out and extracted with 1 ml of dichloromethane (Frutarum, Haifa, Israel) by rough-mixing for 1 min. The organic phase was subjected to SM analysis. While animals were anesthetized, SM in the skin surrounded by the well was extracted by the addition of 1 ml of dichloromethane into the well. To obtain maximal extraction from the skin, the organic solvent was pipetted up and down 3–4 times, then subjected to SM analysis.

As will be shown under the Results section, low SM levels were found in the skin, while both the iodine and vehicle extracts were below the detection level. Therefore, we employed an additional iodine formulation, B, that had protecting properties, although weaker than those of TG-containing preparations (data not shown). The time schedule was extended in order to determine if, after a long period of incubation, iodine would affect SM levels.

One milliliter of iodine formulation B or vehicle B was added to the well 20 min after SM exposure. The iodine solution was aspirated out 2 h later and extracted with dichloromethane. The skin was also extracted as described above. SM in dichloromethane samples was quantified by gas chromatography/mass spectrometry (GC/MS) analysis using appropriate standards. Each



FIG. 1. Macroscopic appearance of the protective effect of iodine against SM-induced skin lesions. Six sites of a shaved guinea pig back were exposed to 1.27 mg (1 μ l) neat SM. The three left-side sites were treated topically with iodine formulation A 30 min after exposure. Photograph was taken 2 days after treatment.

specimen (5 μ l) was injected into an HP 5980 II gas chromatography system equipped with an electron ionizing detector, HT1 30-m capillary column and HP 5971A MS detector. The elution method entailed starting at 100°C for 2 min then increasing the temperature at the rate of 10°C/min up to 200°C. To test the pure effect of iodine on SM, analysis of iodine treatment was compared to that obtained by the appropriate iodine vehicle.

Histopathological evaluation. Each skin section was evaluated in a blinded way, without knowing the identity of the treatment group, and scored for histopathological changes. The reactive and inflammatory changes in the epidermis and dermis were assigned a severity grade of 0–4 representing unremarkable, minimal, mild, moderate, and marked changes, respectively. Epidermal parameters included microblister formation, ulceration, necrosis, crust formation, hyperkeratosis, and acanthosis (epidermal hyperplasia). Dermal parameters included hemorrhage, inflammation (acute and subacute), necrosis, and fibrosis. In addition, the area of epidermal acanthosis was estimated using the grade of 0–6 as follows: Grade 0, no acanthosis; 1, less than 1/3 of the epidermal area acanthotic, the remainder necrotic; 2, about 1/3 acanthotic, the remainder necrotic; 3, about 1/2 acanthotic, the remainder necrotic; 4, less than 2/3 (but more than 1/2) acanthotic, the remainder necrotic; 5, more than 2/3 acanthotic, the remainder necrotic; 6, diffuse acanthosis (entire epidermal area acanthotic).

Quantitative evaluation. To quantify the protective effect of the iodine formulation against SM, we evaluated 57 skin specimens exposed to SM only, 9 treated with iodine 15 min after exposure, and an additional 18 with a 30-min interval between exposure and iodine treatment. Skin specimens were collected 2 days after treatment. In an additional series of experiments with 45- ($n = 15$) and 60- ($n = 15$) min intervals between exposure and treatment and a concurrent control group (SM only, $n = 53$), animals were sacrificed 6 days after treatment. Results are expressed as the mean \pm SE using the Kruskal-Wallis test and Dunnett's multiple comparison post test for statistical compar-

ison of the effects of SM and iodine at different time intervals between exposure and treatment.

RESULTS

Gross pathology findings. The macroscopic appearance of a guinea pig exposed to SM and treated topically with iodine 30 min later (Fig. 1) demonstrates the therapeutic effect of iodine in two out of three treated sites. Evaluation of a series of experiments revealed statistically significant reductions of 91 and 84% in the grossly ulcerated area at intervals of 15 and 30 min between exposure and treatment, respectively (Fig. 2). In order to rule out dilution effect of SM, we show that the iodine vehicle formulation had a weak therapeutic effect (32% reduction in ulceration) which was not statistically different from the exposed, untreated sites.

Histopathological findings. The histopathological findings are illustrated in Fig. 3 and quantified for various treatment groups in Figs. 4 and 5. At the interval of 15 min between exposure and treatment, a statistically significant reduction of 48, 50, and 55% was observed in dermal parameters indicative of acute tissue damage such as acute inflammation, hemorrhage, and necrosis, respectively (Fig. 4, lower profile). Moreover, the epidermal healing markers, acanthosis and hyperkeratosis, were significantly increased by 67 and 72%, respectively (Fig. 4, upper profile). These healing parameters are the most reliable histological proof for the effectiveness of iodine (Figs. 3A–3D). At the longer interval of 30 min between SM exposure and iodine treatment, a significant degree of therapy was conferred, albeit to a lesser extent than that observed in the shorter interval (Figs. 3E, 3F, and 4). Although

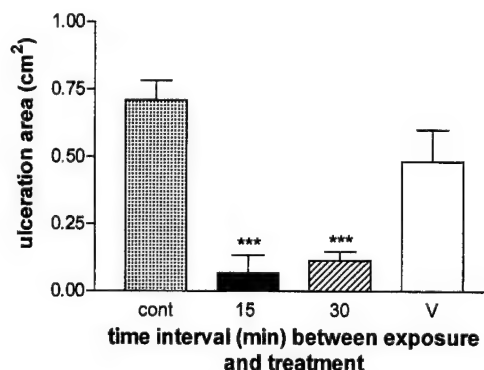


FIG. 2. Gross pathology quantification of the protective effect of iodine. Guinea pigs were exposed to 1.27 mg SM (1 μ l) and were treated topically with iodine formulation A. The ulcerated area was measured 2 days following exposure. The time intervals between exposure and treatment were 15 ($n = 9$) and 30 ($n = 18$) min as indicated. Cont indicates control, SM-exposed skin without iodine treatment ($n = 57$), and V represents vehicle A applied 15 min after SM exposure ($n = 9$). Results are expressed as mean \pm SE using the Kruskal-Wallis test and Dunnett's multiple comparison posttest for statistical evaluation of the differences between the experimental groups. *** $p < 0.001$ at comparison between 15- and 30-min interval and cont. $p < 0.05$ at comparison between 15-min interval and vehicle.

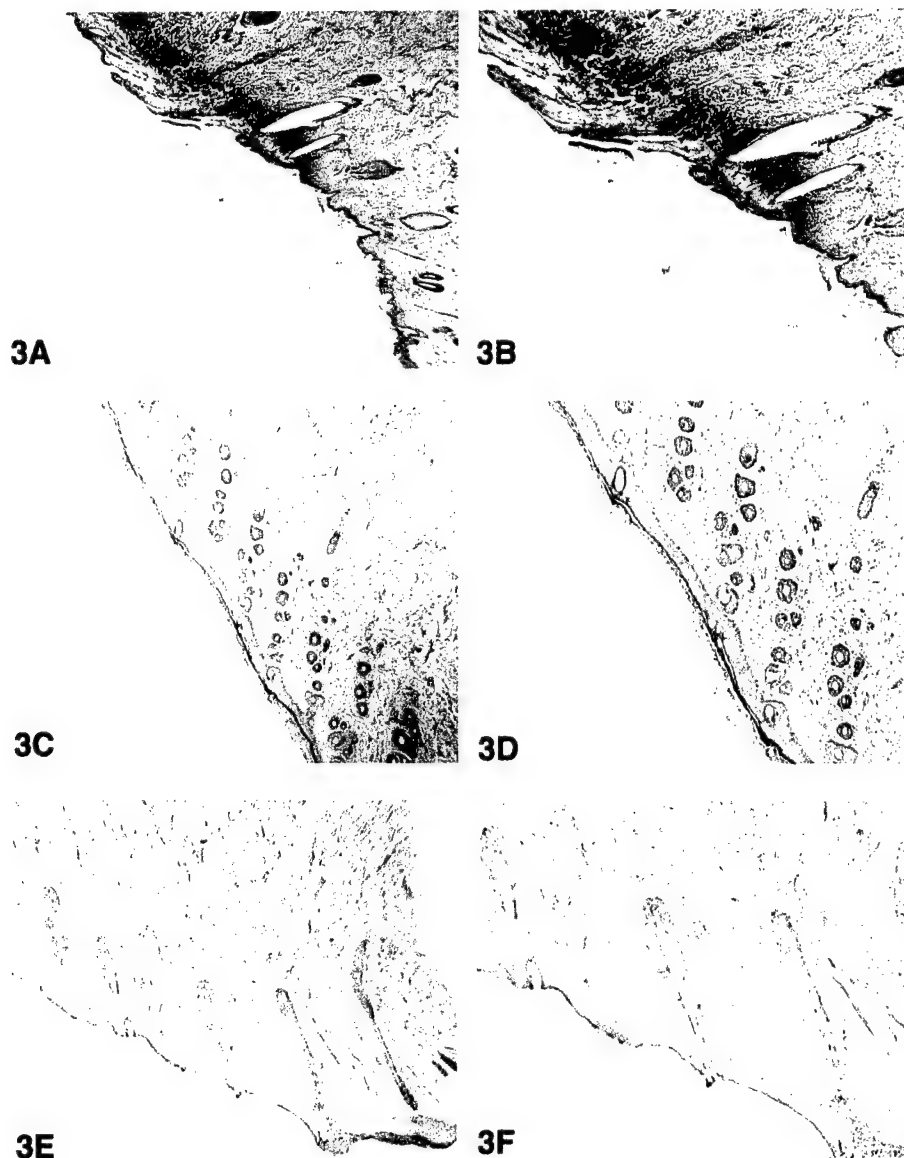


FIG. 3. Histopathology of skin exposed to SM with and without iodine treatment. Photomicrographs of skin samples after exposure to SM (3A, 3B) followed by iodine (formulation A) treatment 15 (3C, 3D) and 30 (3E, 3F) min after exposure. Specimens collected 2 days after exposure. (A, B) Marked epidermal necrosis and ulceration associated with epidermal encrustation, dermal necrosis, and acute inflammation (A: $\times 200$, B: $\times 400$). (C, D) Moderate epidermal hyperplasia and lack of epidermal or dermal ulceration or necrosis (C: $\times 200$, D: $\times 400$). (E, F) Minimal to moderate epidermal hyperplasia, sloughing of epidermal encrustation, and lack of epidermal or dermal ulceration and necrosis (E: $\times 200$, F: $\times 400$).

the epidermal healing markers were not elevated, the parameters indicative of acute tissue damage such as subepidermal microblister formation, epidermal ulceration, and dermal markers including acute inflammation, hemorrhage, and necrosis were significantly reduced by 35, 67, 43, 39, and 45%, respectively (Fig. 4). Iodine had weaker therapeutic activity at the 45-min interval between exposure and treatment (Fig. 5). A degree of protection was expressed by a statistically significant reduction in dermal subacute inflammation, subepidermal microblister formation, and epidermal ulceration, whereas, the regenerative marker, epidermal acanthosis, was significantly increased. At the interval of 60 min between exposure and

treatment, iodine was less effective; nevertheless, a significant reduction in subepidermal microblisters and an increase in the acanthotic area were observed even at this interval.

In vivo effect of iodine on chemical inactivation of SM. The primary question regarding the mechanism of the therapeutic effect of iodine is whether SM is chemically inactivated by iodine in the skin of the living guinea pig. In order to address this issue, animals, after exposure to SM, were treated with liquid iodine preparations or their vehicles in the well system described under Materials and Methods. SM extracted from the skin and from iodine preparations or their vehicle was

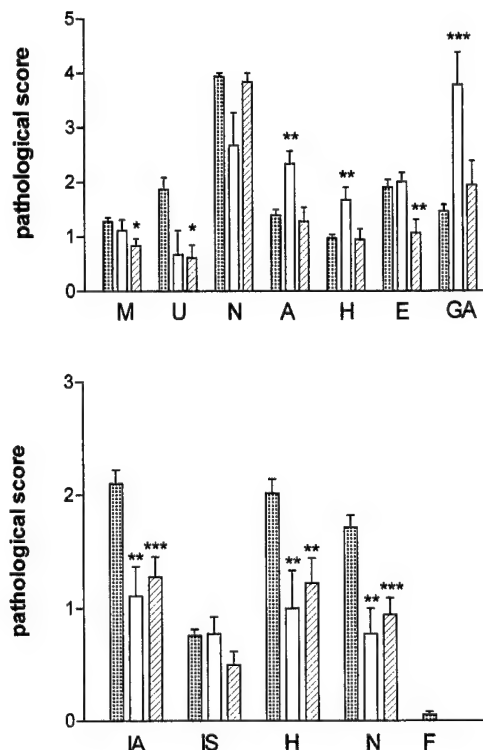


FIG. 4. Histopathology quantification of the protective effect of iodine at intervals of 15 and 30 min between exposure and treatment. Skin sections (stained with H&E) from guinea pigs exposed to 1.27 mg SM (1 μ l), treated topically with iodine (formulation A) and sacrificed 2 days after treatment. Twelve parameters were determined, seven epidermal (upper part) and five dermal (lower part). The epidermal markers were subepidermal microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), and grade of area of epidermal acanthosis (GA). The dermal markers were acute inflammation (IA), subacute inflammation (IS), hemorrhage (H), necrosis (N), and fibrosis (F). The following types of treatment were evaluated: skin exposed to SM only (dotted bars, $n = 57$), skin exposed to SM followed by iodine treatment 15 min (open bars, $n = 9$) or 30 min (hatched bars, $n = 18$) later. Results are expressed as means \pm SE using Kruskal-Wallis test and Dunnett's multiple comparison posttest for statistical evaluation of the differences between controls (SM only) and 15- or 30-min interval between exposure and treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

quantitatively determined by GC/MS analysis (Fig. 6). Two iodine formulations and their vehicles were tested. A slight iodine-induced SM reduction of 36 and 22%, in comparison to the vehicles, was observed after vesicant extraction from skin treated with formulations A and B, respectively (Fig. 6). No iodine-induced SM inactivation was observed after extraction of SM from the liquid iodine preparations (Fig. 6).

Effect of lidocaine on the therapeutic effect of iodine. Because previous studies have shown that neuronal activity is involved in the evolution of skin damage caused by chemical irritants (Veronesi *et al.*, 1995), we tested the effect of the local anesthetic lidocaine on both skin toxicity of SM and the protective effect of iodine (Fig. 7). Local anesthesia affected neither the area of skin ulceration nor the protective benefits of iodine, when compared to saline-treated controls.

DISCUSSION

The present study demonstrates the therapeutic effect of the newly developed TG-containing iodine formulation against sulfur mustard-induced skin lesions. These data prove the superiority of the present iodine formulation over the previously used povidone iodine preparation (Wormser *et al.*, 1997) that was efficacious only at short intervals of 5 and 10 min, and to a much lesser extent at 20 min, between exposure and treatment. The fact that significant therapeutic effect can be achieved at intervals of 15 and 30 min between SM exposure and iodine treatment strongly emphasizes the usefulness of our iodine preparation, over the povidone iodine, as the preferred antidote against a chemical attack of SM. Moreover, the fact that longer intervals of 45 and 60 min have some beneficial effect, although weaker than that of the shorter intervals, further supports the usage of the topical iodine preparation as a pharmacological countermeasure against SM in emergencies.

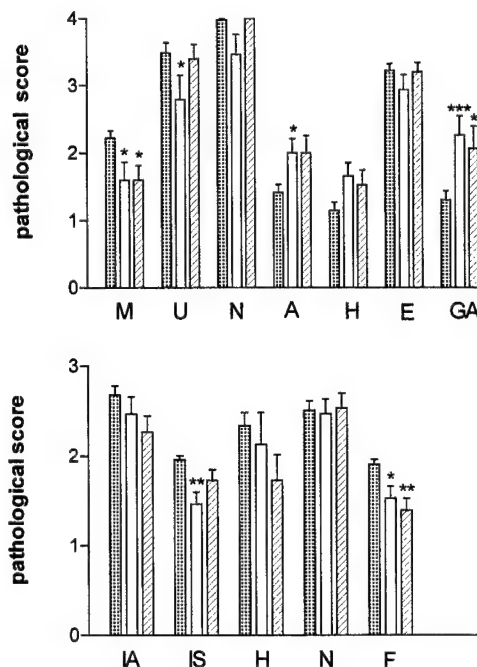


FIG. 5. Histopathologic quantification of the protective effect of iodine at intervals of 45 and 60 min between exposure and treatment. Skin sections (stained with H&E) from guinea pigs exposed to 1.27 mg SM (1 μ l), treated topically with iodine (formulation A) and sacrificed 6 days after treatment. Twelve parameters were determined, seven epidermal (upper part) and five dermal (lower part). The epidermal markers were subepidermal microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), and grade of area of epidermal acanthosis (GA). The dermal markers were acute inflammation (IA), subacute inflammation (IS), hemorrhage (H), necrosis (N), and fibrosis (F). The following types of treatment were evaluated: skin exposed to SM only (dotted bars, $n = 53$), skin exposed to SM followed by iodine treatment 45 min (open bars, $n = 15$) or 60 min (hatched bars, $n = 15$) later. Results are expressed as mean \pm SE using Kruskal-Wallis test and Dunnett's multiple comparison posttest for statistical evaluation of the differences between controls (SM only) and 45- or 60-min interval between exposure and treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

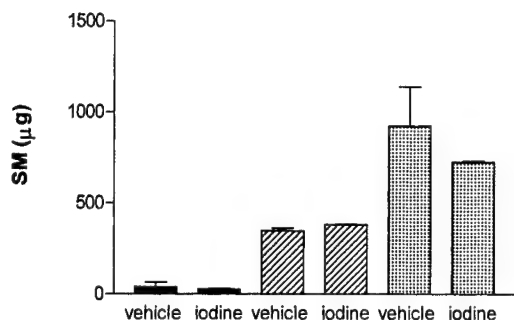


FIG. 6. Effect of iodine on SM inactivation *in situ*. Iodine formulation A or vehicle A were applied 10 min following SM (1 μ l neat liquid). Twenty minutes later the iodine solution and the skin were extracted for SM analysis with dichloromethane. Low amounts of SM were detected in the skin (closed bars) after treatment with iodine formulation A or vehicle A. No SM could be detected in the iodine solution or its vehicle (not shown). An additional procedure included application of 1 ml iodine formulation B or vehicle B 20 min following SM exposure. Two hours later the iodine or vehicle solution (hatched bars) and the skin (dotted bars) treated with iodine or its vehicle were extracted with dichloromethane for SM analysis. Results are the mean of three experiments. Iodine formulations and extraction methodologies are described under Materials and Methods.

Numerous iodine formulations were tested, but none was proven to be as efficacious as the one containing TG as solvent. The superiority of this iodine formulation stems from its ability to dissolve molecular iodine in an aqueous environment. Molecular iodine (I_2) is practically water-insoluble unless iodide (sodium or potassium salts) is present in the solution (Reynolds, 1993) to form the water-soluble ion I_3^- . Molecular iodine can be dissolved in organic solvents such as ethanol or polyethyleneglycol-400 (PEG-400), but the presence of water precipitates the iodine; thus, iodine tincture which contains ethyl alcohol and water must also contain iodide in the form I_3^- for proper dissolution. Experiments conducted in our laboratory showed that iodine tincture exhibited a weaker protective effect than the iodine formulation described in the present study (data not shown). A possible explanation is that the negatively charged I_3^- penetrates poorly through biological membranes and barriers, thus reducing its efficacy as a protectant. The TG-containing solvent system that dissolves I_2 without the addition of iodide, thus keeping the molecular iodine in its noncharged form, I_2 , might be more penetrable and a stronger oxidizer than the negatively charged I_3^- and more efficient in its protecting activity. This explanation may also account for the superiority of the TG-containing formulation over the iodide-containing formulations for therapy against thermal burns and in its bactericidal effect (data not shown). Such an assumption must be experimentally proven by physicochemical and biochemical experiments.

Although the functioning of the iodine formulation requires elucidation, the main issue to be addressed concerns the mechanism of the protective action of iodine. Since iodine does not chemically inactivate SM (Fig. 6), the protective effect may originate from epidermal/dermal processes affected by iodine.

Moreover, that postexposure treatment with iodine is also effective against thermal burns (Wormser, 1998) further demonstrates the possible epidermal/dermal origin of its antidotal activity. Since local anesthesia did not alter the protective effect of iodine against SM (Fig. 7), the antidotal effect likely does not directly involve sodium channel-dependent neuronal activity.

Increasing evidence indicates that exposure to irritants is associated with the tendency of tissue to undergo programmed cell death, namely, apoptosis. Apoptotic cells were demonstrated in cultured keratinocytes (Rosenthal *et al.*, 1998), endothelial cells (Dabrowska *et al.*, 1996), and thymocytes (Hur *et al.*, 1998; Michaelson, 2000) after exposure to SM or its derivatives. *In vivo* studies have shown the appearance of apoptotic cells in SM-exposed skin of weanling pigs (Smith *et al.*, 1997). The apoptotic process comprises a variety of biochemical reactions among which the activity of the cysteine proteinases, caspases, plays an important role (Asahi *et al.*, 1999; Hashimoto *et al.*, 1998). We hypothesize that iodine exerts its protective activity by inhibition of apoptotic processes, namely, by oxidizing sulfhydryl groups of either the active site of caspases or other functional proteins or peptides crucial for apoptosis. This hypothesis is currently under investigation.

Although the mechanism of iodine-induced therapy must be elucidated, the present study demonstrates the usefulness of our iodine formulation as a potent antidote against skin lesions caused by SM. We propose that this type of topical preparation can be used as a protectant during both military and civilian emergencies.

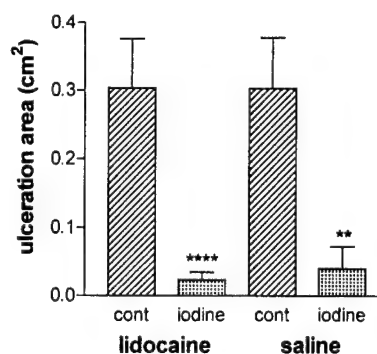


FIG. 7. Effect of lidocaine on the protective effect of iodine. The skin area was locally anesthetized with lidocaine (2%, 0.1 ml) injected sc about 1 cm laterally to each site of exposure so that each animal received six injections. For comparison, control animals were injected with saline. Twenty minutes later, six sites on each animal back were exposed to 1.27 mg neat SM (marked as cont, hatched bars). Iodine formulation C ointment was topically applied for 15 min (dotted bars). Lidocaine or saline treatment was repeated 2 h after the first injections. Ulcerated areas were measured 7 days after exposure. Results are expressed as mean \pm SE using the Mann-Whitney two-tailed test for statistical evaluation of the differences between (a) the control (SM only, $n = 21$) and iodine treatment ($n = 21$) of the lidocaine-injected animals (**** $p < 0.0001$); (b) the control ($n = 18$) and iodine treatment ($n = 15$) of the saline-injected animals (** $p < 0.01$).

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Effects of iodine on inducible nitric oxide synthase and cyclooxygenase-2 expression in sulfur mustard-induced skin injury in guinea pigs

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Abstract In a previous study we demonstrated the protective effect of topical iodine as postexposure treatment for sulfur mustard (SM) application. The iodine treatment results in significantly reduced inflammation and necrosis and increased epidermal hyperplasia. The expression and localization of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in paraffin-embedded skin samples from that study were evaluated in the present investigation. We compared the immunoreactivity of iNOS and COX-2 using five samples from each of the following four test sites: untreated control sites, SM-exposed sites, sites treated with iodine mixture 15 min after SM exposure, and sites treated with iodine 30 min after SM exposure. All animals were killed 2 days after irritant exposure. iNOS immunoreactivity was present only in skin sites exposed to SM without iodine treatment. The ulcerated skin was covered with a relatively thick band of exudate composed of iNOS-immunostained polymorphonuclear cells and macrophages. In untreated skin, COX-2 immunostaining was limited to the thin suprabasal epidermal layer. In SM-

exposed skin, induction of COX-2 was noted in inflammatory cells located close to the site of epidermal injury. In skin sites treated with iodine 15 or 30 min after SM exposure, the regenerating hyperplastic epithelium showed moderate cytoplasmic staining localized to the epithelium overlying the basal layer. This pattern of staining was also present in the nearby dermal fibroblasts. Thus, in contrast to the skin samples exposed to SM without iodine treatment, the epidermal layer expressing immunohistochemical positivity for COX-2 was thicker and corresponded to the epidermal hyperplasia noted in samples treated with iodine. It is well documented that prostaglandins (PGs) promote epidermal proliferation, thereby contributing to the repair of injured skin. That the induction of the COX-2 shown in our study may also play a role in the healing process is indicated by the present evidence. The results suggest that nitric oxide radicals (NO·) are involved in mediating the damage induced by the SM and that iodine-related reduction in acute epidermal inflammation is associated with reduced iNOS expression.

Key words Sulfur mustard · Skin · COX-2 · iNOS

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Introduction

Sulfur mustard (SM), a powerful vesicant or blister agent employed in chemical warfare, possesses strong alkylating properties and consequently demonstrates systemic toxicity in addition to its localized effects on skin, eyes, and respiratory tract (Munro et al. 1999; Wormser 1991). Our recent findings suggest that a topical iodine preparation is a potential antidote for skin lesions induced by SM. This is expressed as a significant reduction in inflammation and necrosis and, at the same time, hyperplasia of the epidermis in the SM guinea pig skin model (Wormser et al. 2000).

Inflammation, a complex process, involves numerous mediators of cellular and plasma origin with interrelated biological effects (Vane et al. 1994). Prostanoids and

nitric oxide (NO \cdot) which are released by inflammatory cells are mediators implicated in all stages of inflammation. The free radical nitrogen species (NO \cdot) is synthesized from L-arginine by the action of nitric oxide synthase (NOS) (Moncada et al. 1991). Of the known families of NOS-related enzymes, the isoform inducible NOS (iNOS) is found predominantly in mononuclear phagocytes and neutrophils, and its expression is modulated by various cytokines (McCafferty et al. 1999; Sharara et al. 1997). The role of (NO \cdot) in inflammation is complicated by reports of both pro- and antiinflammatory actions (Vane et al. 1994). Epidermal keratinocytes in normal skin contain both endothelial NOS and iNOS (Paulsen et al. 1998; Shimizu et al. 1997).

Cyclooxygenase (COX) is the enzyme that catalyzes the formation of prostaglandins (PGs) and other eicosanoids from arachidonic acid. Two major forms of COX occur – COX-1 and COX-2. COX-1 is constitutively expressed in epithelial and mesenchymal cells and functions in normal cell physiology (Fosslien 2000; Khan et al. 1998). COX-2 is inducible and is expressed in a number of cells such as mononuclear phagocytes and neutrophils by proinflammatory stimuli leading to the synthesis of PGs, with suggested pro- and antiinflammatory activities varying according to the phase of inflammation (Gilroy et al. 1999; Minghetti et al. 1999). The immunohistochemical expression of COX-2 has been compared between human and mouse skin (Leong et al. 1996). In human skin, COX-2 immunostaining increases in the more differentiated, suprabasal keratinocytes, in contrast to lack of expression of this enzyme in the same layers in the mouse. It was then suggested that the expression of COX-2 in human epidermis occurs as part of normal keratinocyte differentiation.

Inhibition of PG formation is the generally accepted therapeutic basis for intervention with nonsteroidal antiinflammatory drugs. That PGs such as PGE₂ and PGF_{2 α} may be crucial mediators involved in the repair of injured epidermis has also been suggested, but the regulatory mechanism remains unclear (Sato et al. 1997). In an effort to understand some aspects by which an iodine solution protects the skin from SM damage, we applied immunohistochemical markers to investigate the

expression and localization of iNOS and COX-2. Skin samples were obtained from our previously described experiment in guinea pigs exposed to SM and treated topically with iodine (Wormser et al. 2000).

Materials and methods

Test materials

SM (generic name 2',2-dichlorodiethylsulfide) was prepared as described previously (Wormser et al. 1997). Briefly, SM was synthesized by reaction of 2',2-thiodiethanol (Sigma, St. Louis, Mo.) with concentrated HCl (Merck, Darmstadt, Germany). Iodine (Merck) (2%) was prepared in a 1:1 mixture of tetraglycol (Sigma) and water.

Animals

Male Dunken Hartley guinea pigs supplied by Harlan Laboratories Breeding Center (Ein Karem, Jerusalem, Israel) weighing 650–850 g were used. Animals were acclimatized for a period of 7 days to ensure their suitability for the study. Test animals were kept within a limited-access rodent facility with environmental conditions set to a temperature of 20 \pm 2 $^{\circ}$ C, a humidity of 30–70% and a 12-h light/12-h dark cycle. Temperature and relative humidity were monitored daily. The animals were housed in filter-covered polypropylene cages with solid bottoms covered with wood shavings as bedding material. Animals were provided ad libitum access to a commercial rodent diet (Kofolk 19520, Netanya, Israel), and drinking water was supplied to each cage via propylene bottles with stainless steel sipper tubes. All procedures, maintenance, and treatment of the animals were in accordance with the principles of humane treatment published by the National Academy of Sciences (US Department of Health and Human Services 1996). The Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem approved the protocol.

Study design and experimental procedure

The different testing forms are presented in Table 1. The backs of the animals were shaved 24 h prior to the experiment. The animals were anesthetized by intraperitoneal (i.p.) injection of 30 mg/kg pentobarbital sodium. The backs were cleaned with wet soft white paper and allowed to dry before the beginning of the experiment. Six sites (three on each side) on each back were exposed to 1 μ l (1.2 mg) SM. Three exposure sites of each animal were treated with 1 ml of liquid iodine preparation, while the other three SM-exposed sites of each animal remained untreated. Wells to contain the iodine

Table 1 Experimental study design and results of iNOS and COX-2 immunohistochemical scoring. Samples were collected 2 days after exposure to SM. All samples were checked histologically and have been described previously (Wormser et al. 2000). The values indicate immunohistochemical staining scored from 0 to 4 as

follows: 0 – no specific immunohistological reaction visible in any of the cells, 1 – minimal, 2 – slight, 3 – moderate, 4 – marked specific reaction. A uniform pattern of staining reaction was noted in all five skin samples from each treatment (*N* tissue component not present)

Treatment ^a	Epidermal exudate		Epidermal suprabasal layer		Dermal fibroblasts	
	iNOS	COX-2	iNOS	COX-2	iNOS	COX-2
Control	N	N	0	3	0	0
SM	1–2	1–2	N	N	0	0
SM + iodine 15 min later	0	0	0	3 ^b	0	3
SM + iodine 30 min later	0	0	0	3 ^b	0	3

^a Five samples from each treatment

^b The epidermal layer expressing immunohistochemical positivity was thicker than that seen in the control sites and corresponded to the epidermal hyperplasia noted in that site

solution were constructed by cutting a plastic tube cover (inner diameter of 1.7 cm) to form an open-ended cylinder. A thin layer of commercial silicon sealing ointment was applied to one edge of each well to provide an impervious seal, and the wells were then attached to the animals' backs so that the liquid inside could not leak out. In all cases of iodine treatment, wells were constructed before exposure, and SM was applied in the center of the wells. Wells were filled with iodine 15 and 30 min after SM exposure, and the preparation was left on the skin for 2 h. At the end of the procedure, the iodine mixture was withdrawn and the well removed. Control sites consisted of skin areas that were exposed to neither SM nor iodine.

Tissue preparation and histological examination

All test animals were killed by injection of 100 mg/kg pentobarbital sodium i.p. 48 h following SM exposure. A complete necropsy was performed on all animals. The skin samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5–6 μ m, and stained with hematoxylin and eosin for histopathological evaluation. Lesions were described and scored where appropriate using five semiquantitative grades as follows: 0 – no staining, 1 – minimal, 2 – mild, 3 – moderate, 4 – severe.

Immunohistochemical investigation

For the immunohistochemical evaluations, paraffin-embedded skin samples from the following sites were used (Table 1): control sites, SM-exposed sites, sites treated with the iodine mixture 15 min after SM exposure, and sites treated with iodine 30 min after SM exposure. All animals were killed 2 days after the irritant exposure. Immunohistochemistry was performed using the avidin-biotin-peroxidase method. Sections were stained with one of the following antibodies: anti-iNOS (Transduction Laboratories, Lexington, Ky.) or COX-2 (Cayman Chemical, Ann Arbor, Mich.). Following deparaffinization through xylene and a series of graded alcohols, all slides were placed in 1 \times automation buffer (AB) (Biomedex, Foster City, Calif.) and blocked for endogenous peroxidase activity with 3% H₂O₂ for 15 min. Heat-induced epitope retrieval was completed for all slides by incubating the slides in 1 \times citrate buffer, pH 6.0 (Biocare Medical, Walnut Creek, Calif.). Slides stained for iNOS were placed in a Decloaker (Biocare Medical) for 5 min and cooled for 10 min following depressurization. Slides stained for COX-2 were heated for 5 min in a microwave oven at 50% power for two cycles. Between cycles, 50 ml fresh citrate buffer was added to the Tissue-Tek (Sakura, Torrance, Calif.) container. After microwaving, the slides were allowed to cool for 15 min, then rinsed in distilled water and placed in 1 \times AB.

All incubations were carried out in a humidified chamber at room temperature. Slides for iNOS and COX-2 were blocked in 5% normal goat serum (Jackson ImmunoResearch, West Grove, Pa.) and then incubated with the respective primary antibody, iNOS or COX-2, each at a dilution of 1:300, for 1 h. Normal rabbit serum (Jackson ImmunoResearch) was used as the negative control. For the iNOS staining, the positive control was a macrophage cell line derived from a mouse and stimulated with lipopolysaccharide (LPS). For the COX-2 staining, the positive control was a superovulating mouse subcutaneously injected with 5 IU of pregnant mare serum gonadotrophins (PMSG) followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG), and killed 8 h later. Slides were washed in buffer, incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif.) for 30 min, washed again, and then labeled with an avidin-biotin complex (Vector Elite Kit, Vector Laboratories) for 30 min. Slides were rinsed in water, counterstained with Harris hematoxylin (Hareco, Gibbstown, N.J.) and coverslipped with Permount (Surgipath, Richmond, Ill.). The immunohistochemical staining of the sections was scored without knowledge of the identity of the tested site ("blind evaluation"). A scale ranging from 0 to 4 was used as follows: 0 – no specific immunohistological

reaction visible in any of the cells, 1 – minimal, 2 – slight, 3 – moderate, 4 – marked specific reaction.

Results

Histopathological findings

Histological changes have been reported previously (Wormser et al. 2000). In brief, a statistically significant reduction in acute epidermal inflammation, hemorrhage, necrosis and a significant elevation in epidermal healing markers such as hyperkeratosis and acanthosis were noted when the iodine was applied 15 min after SM exposure. With the longer interval of 30 min between SM exposure and iodine treatment there was a significant degree of protection, albeit to a lesser extent.

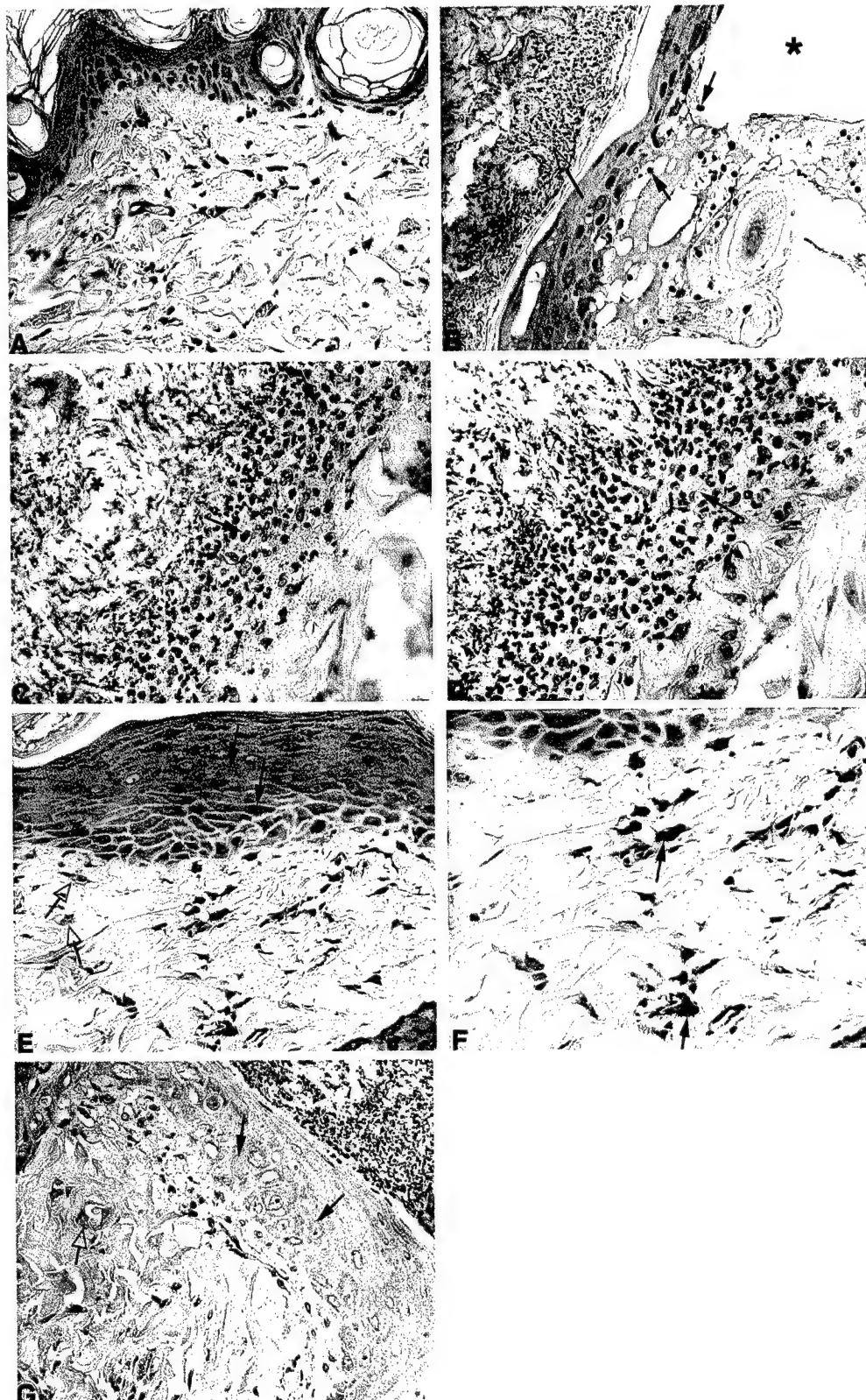
Staining for COX-2

A uniform pattern of immunohistochemical reaction was noted in all five skin samples from each of the four test sites examined (Table 1). A moderately positive reaction was noted in the untreated control skin, limited to the cytoplasm of the epithelial cells in the suprabasal layers of the epidermis (Fig. 1A). No immunostaining was noted in the dermal fibroblasts. At skin sites exposed to SM without iodine treatment of animals killed 2 days later, grade 4 immunostained dense granules were noted within the necrotic epidermal cells surrounding the subepidermal vesicles (Fig. 1B). These granules

Fig. 1A–G Representative photomicrographs demonstrating the expression and localization of COX-2 protein in the skin of male guinea pigs. **A** Untreated control site. Immunostaining is present in the suprabasal keratinocytes (arrow). The expression of COX-2 occurs as part of normal keratinocyte differentiation ($\times 60$). **B** Photomicrograph of a site exposed to SM of an animal killed 2 days later. Note the presence of strongly immunostained dense granules (dark arrows) within necrotizing epidermal cells, surrounding a subepidermal vesicle (asterisk). The granules have a hyaline appearance in hematoxylin and eosin-stained sections (open arrow necrotic cell exudate) ($\times 60$). **C** Photomicrograph of a site exposed to SM of an animal killed 2 days later. Note the presence of moderately immunostained polymorphonuclear cells and macrophages (arrows) at the site of epidermal ulceration. Note the negative immunostaining overlying necrotic cell exudate (asterisks) ($\times 40$). **D** Photomicrograph of a site exposed to SM of an animal killed 2 days later. Same site as shown in C; negative control using normal rabbit serum instead of COX-2 antibody ($\times 60$). **E** Photomicrograph of a site treated with iodine 15 min after SM exposure; animal killed 2 days after the irritant exposure. The epidermis is moderately hyperplastic. Immunostaining is present in the suprabasal keratinocytes (dark arrows). Open arrows indicate immunoreactivity in the dermal fibroblasts ($\times 60$). **F** Photomicrograph of a site treated with iodine 15 min after SM exposure; animal killed 2 days after the irritant exposure (higher magnification of E). Note moderately immunostained dermal fibroblasts (arrows) ($\times 80$). **G** Photomicrograph of a site treated with iodine 30 min after SM exposure; animal killed 2 days after the irritant exposure. The epidermis is moderately hyperplastic. Immunostaining is present in the suprabasal keratinocytes (dark arrows) and in scattered dermal fibroblasts (open arrow) ($\times 60$).

showed a hyaline structure in hematoxylin and eosin-stained sections. The ulcerated SM-exposed skin was covered with a relatively thick band of exudate composed of grade 1 or 2 immunostained polymorphonuclear (PMNL) cells and macrophages. The outer

necrotic exudate did not show any positivity for COX-2 (Fig. 1C, D), but in skin sites treated with iodine 15 min after SM exposure, the regenerating, hyperplastic epithelium showed moderate cytoplasmic staining localized to the epithelium overlying the basal layer (Fig. 1E).



Thus, in contrast to the skin samples exposed to SM without iodine treatment, the epidermal layer expressing immunohistochemical positivity for COX-2 was thicker and corresponded to the epidermal hyperplasia noted in the iodine-treated sites. Moderate cytoplasmic immunostaining was present also in adjacent sparse dermal fibroblasts and macrophages (Fig. 1E, F), as well as in PMNL cells and macrophages delineating subepidermal vesicles or ulcers. A similar pattern and intensity of cellular immunostaining was observed in the samples treated with iodine 30 min after SM exposure (Fig. 1G).

Staining for iNOS

A uniform pattern of immunohistochemical reaction was noted in all five skin samples from each of the four test sites examined (Table 1). iNOS immunoreactivity was present only in inflamed skin sites exposed to SM without iodine treatment of animals killed 2 days later. At these sites, the ulcerated skin was covered with a relatively thick band of exudate composed of cytoplasmic grade 1 or 2 immunostained PMNL cells and macrophages. The outer necrotic exudate did not show any positivity for iNOS. No iNOS immunoreactivity was detected in any of the dermal cells.

Discussion

PGs and (NO \cdot), the byproducts of COX-2 and iNOS, respectively, can have both beneficial and deleterious effects on tissue inflammation and injury (Fu et al. 1999). Conflicting results or interpretations indicating proinflammatory and antiinflammatory effects have been related to the increased expression of COX-2 and iNOS in epithelial cells, as in gastrointestinal mucosal inflammation (Eckmann et al. 1997; Fu et al. 1999). It has been suggested that (NO \cdot) radicals limit PMNL infiltration into the mucosa, while prostaglandin E₂ (PGE₂) is believed to facilitate wound healing by inhibiting T-cell proliferation and production of T helper (Th1) cytokines and activating production of Th2 cytokines (Fu et al. 1999). On the other hand, a mitigation of LPS-induced hepatocellular toxicity has been shown in animals lacking COX-2, suggesting that PG synthesis by inflammatory cells contributes significantly to the cytotoxicity of LPS (Dinchuk et al. 1995). Studies using different inflammatory models have suggested that the change in the expression and role of iNOS and COX-2 with the progression of inflammation is related to kinetic changes in the profile of cytokines (Bailey and Verma 1991; Gilroy et al. 1999; Minghetti et al. 1999; Schini et al. 1992; Vane et al. 1994). The interaction of (NO \cdot) directly with COX to cause an increase in enzymatic activity has also been suggested (Salvemini et al. 1993).

The cellular distribution of iNOS is considered a reliable marker for sites of (NO \cdot) production (Paulsen et al. 1998). The localized skin production of iNOS has

been suggested to be a direct function of the cutaneous response to cytokines (Bruch-Gerharz et al. 1998). Human skin burn wounds show an upregulation in iNOS expression in keratinocytes located close to the wound, including those in the newly surfacing epidermis, immediately after injury and during the prolonged reparative phase of wound healing (Paulsen et al. 1998). These findings support the notion that (NO \cdot), acting either in a paracrine or autocrine manner, play a role in the initial inflammatory response to burn injury and also have a role in epidermal proliferation within healing wounds. The (NO \cdot) molecule is associated with the hemodynamic and immunological alterations observed after thermal injury (Preisner et al. 1996). Paulsen et al. (1998) have suggested that the macrophages are a potential source of (NO \cdot) production in the burn wound, being an effector molecule in the nonspecific defense mechanisms.

Our observation of iNOS expression only in inflammatory cells at the skin sites exposed to SM without iodine treatment, supports the involvement of (NO \cdot) radicals in promoting the cellular damage induced by SM, or indicate its role in boosting the nonspecific defense needed in the breached skin. The reduced iNOS expression observed in skin treated with iodine may be related to the iodine-reduced numbers of acute inflammatory cells. Expression of COX-2 has been demonstrated in inflammatory cells in the epidermis at SM-treated sites, but following iodine treatment, the epidermal inflammation decreased, while the epidermal layer expressing COX-2 became thicker, corresponding to the epidermal hyperplasia noted in the iodine-treated sites. This was associated with moderate cytoplasmic immunostaining in adjacent sparse dermal fibroblasts and macrophages.

Various examples documented in the literature demonstrate increased expression of COX-2 and/or increased prostaglandin synthesis in pathological and regenerative processes involving the skin. COX-2 is highly expressed in skin cancer (Buckman et al. 1998; Fosslien 2000). Using celecoxib, a specific COX-2 inhibitor, a dose-dependent reduction in tumor yield has been shown in UV-induced skin tumors in hairless mice. This emphasizes the promotional effect of COX-2 on epidermal cell proliferation (Fischer et al. 1999). It has been suggested that COX-2 is induced by the oncogenes *ras* and *src*, as well as by various factors such as interleukin-1, epidermal growth factor, and tumor necrosis factor alpha (Fosslien 2000). Dexamethasone, antioxidants, and the tumor-suppressor protein p53 suppress COX-2 expression. PGE₂ has been shown to stimulate bcl-2 and to inhibit apoptosis, inducing interleukin-6 and hepatoglobulin synthesis (Fosslien 2000). Increased synthesis of PGE₂ by nonconfluent human keratinocytes is accompanied by an increase in [³H]thymidine incorporation into DNA (Pentland and Needleman 1986). The prostaglandin PGF₂ α has been shown to function as a growth factor in fibroblast cultures, initiating DNA synthesis and proliferation (De Asua et al. 1975). Cell-cell interactions of keratinocytes and fibroblasts aug-

ment the production of PGE₂ by a mechanism in which the activity of COX-2 in fibroblasts is increased by the keratinocyte-derived pro-IL-1 α in a paracrine manner (Sato et al. 1997).

Enhancement of PG production by dermal fibroblasts has been suggested to contribute to the healing process by organizing the extracellular matrices in the epidermal/stromal junction. In studies with essential fatty acid-deficient rats, whose skin does not contain the precursor for PG synthesis, epithelial regeneration and repair following burn or incisional injuries are markedly reduced (Hulsey et al. 1980).

Our findings of increased COX-2 expression in regenerating epidermis and dermal fibroblasts are consistent with those of other studies which have demonstrated that PGE₂ plays a role as a mediator in the repair process of injured skin (Sato et al. 1997). PGE₂ has been found to be a required comitogen for phorbol ester-elicited hyperproliferation or proliferation following mechanical wounding (Furstenberger and Mark 1990). Topical application of PGE₂ to patients with chemotherapy-associated mucosal ulcerations induces a rapid reduction in inflammatory response followed by re-epithelialization of the lesions (Kuhler et al. 1986). Available data concerning the involvement of PGs in epithelial hyperplasia would suggest that iodine treatment is associated with immunopositivity for COX-2, and thus may promote skin regeneration. This newly formed epidermis prevents further progression of the detrimental effects of SM. Whether COX-2 is induced directly by iodine or secondarily to the primary protective effect of iodine is unclear. The protective effect of iodine may also be the result of its antiseptic and antibacterial properties (Fairclough et al. 1986). In future studies, the specificity of iodine will be tested by evaluating the counter-irritating effect of other antiseptic and bacteriostatic agents.

In summary, exposure to SM resulted in the induction of iNOS and COX-2 in inflammatory cells located close to the site of epidermal injury. Treatment with iodine following SM exposure was associated with a decreased number of inflammatory cells, as well as thickening of the epidermis which exhibited COX-2 expression in the thickened suprabasal epithelium and in the nearby dermal fibroblasts. These results indicate that (NO \cdot) radicals may be involved in mediating the damage induced by SM and that the reduction of epidermal acute inflammation is associated with reduced iNOS expression. The results also suggest that the protective effect of iodine on the skin is related to the presence of COX-2 in the suprabasal epithelial region. At present, it is unknown whether iodine directly induces COX-2 expression in regenerating skin tissue, or whether the protective effects of iodine are related to its antiinflammatory effect or some other unknown mechanism(s).

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Topical treatment with povidone iodine reduces nitrogen mustard-induced skin collagenolytic activity

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Abstract Recently we have shown that post-exposure treatment with povidone iodine (PI) protects against nitrogen and sulfur mustard-induced skin lesions. Since proteolytic activity is involved in skin damage caused by chemical irritants, we have studied the effect of iodine on mechlorethamine (HN2)-induced skin collagenolytic activities in the haired guinea pig model. The matrix metalloproteinase-9 (MMP-9) activity increased by 30, 46, 12 and 23% after 3, 24, 48 and 72 h of HN2 exposure, respectively, whereas the MMP-2 was elevated by 8, 65, 8 and 30%, respectively. Topical treatment with PI at 15 and 120 min after HN2 exposure decreased the MMP-9 activity by 67% and 60%, respectively, when skin was analyzed 3 h after exposure. The same trend was observed in the MMP-2 and MMP-1 activities after PI treatment. A stronger effect of PI treatment 15 min following exposure was observed in skin analyzed 24 h after exposure, i.e. a decrease of 83% and 88% in MMP-9 and MMP-2 activities, respectively. Similar findings were observed with an interval of 120 min between HN2 exposure and PI treatment. A much weaker effect was observed on MMP-1 activity. A similar trend of PI-induced reduction in the three types of collagenase activity was found in skin analyzed 48 and 72 h after exposure. Reduced collagenolytic activity may serve as one of the mechanisms by which iodine protects the skin against chemical insult.

Keywords Nitrogen mustard · Iodine · Dermatotoxicity · Collagenase · Matrix metalloproteinase · Skin irritation

Introduction

Sulfur and nitrogen mustards are potent vesicants and powerful alkylators (Somani and Babu 1989; Wormser 1991). The former is used as a chemical weapon whereas the latter is used as an antineoplastic agent. Skin exposure to both mustards causes erythema followed by edema, vesicle and blister formation, ulceration, necrosis and desquamation (Smith and Dunn 1991; Smith et al. 1995a, 1995b). The severity of lesions observed after exposure to mustards emphasized the need for an efficient pharmacological antidote against their vesicating activity in cases of emergencies or accidents (Smith and Dunn 1991; Wormser 1991; Smith et al. 1995b). In order to develop such a countermeasure against vesicating agents it is crucial to understand the cellular and molecular mechanism of these blister-forming agents.

Recent reports raised the possibility that proteolytic activity may be involved in the blistering process caused by sulfur mustard (Cowan et al. 1991, 1992, 1993; Cowan and Broomfield 1993). The fact that epidermis can be experimentally separated from the dermis by treatment with the bacterial proteinase dispase (Sawyer and Risk 2000) further emphasizes this hypothesis. Several ex vivo studies have shown collagenase release from skin organ culture of rabbit and human origin after exposure to sulfur mustard (Woessner et al. 1990; Rikimaru et al. 1991).

Metalloproteinases represent a group of proteolytic enzymes which require zinc and calcium for proper activity. To date, more than twenty members of this family have been characterized and cloned. Cellular matrix metalloproteinase (MMP) production may be regulated by various extracellular agents. These include insoluble factors such as crystals and collagen, cytokines such as interleukin 1, interleukin 8 (Singh et al. 1995;

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Luca et al. 1997), basic fibroblast growth factor (Singh et al. 1997), vascular endothelial growth factor and other hormones. Metalloproteinase activity is modulated at various levels including proenzyme synthesis, secretion and activation or specific inhibition by tissue inhibitors of MMPs. Examples of these phenomena have been illustrated in vitro with a diversity of cells including endothelial cells, fibroblasts, corneal cells and transformed cells. The aim of the present study was to examine the activity of skin collagenases following topical exposure to nitrogen mustard. Since post-exposure treatment with iodine protects skin against mustards (Wormser et al. 1997, 2000a, 2000b), the effect of iodine on collagenase activity was also evaluated.

Materials and methods

Animals

Male guinea pigs (Dunkin-Hartley, 600–800 g) were supplied by Harlan Laboratories Breeding Center (Ein Karem, Jerusalem, Israel). Following an acclimatization period of 7 days to ensure their suitability for the study, animals were randomly assigned to experimental groups. Test animals were kept within a limited-access rodent facility with environmental conditions set to a temperature of $20 \pm 2^\circ\text{C}$, a humidity of 30–70% and a 12-h light/12-h dark cycle. Temperature and relative humidity were monitored daily. The animals were housed in filter-covered, solid-bottomed, polypropylene cages with wood shavings as bedding material. Animals were provided with ad libitum access to a commercial guinea pig diet (Kofolk 19520; Netanya, Israel), and drinking water was supplied to each cage via propylene bottles with stainless steel sipper tubes.

All procedures, maintenance, and treatment of the animals were in accordance with the principles of humane treatment published by the National Academy of Sciences (US Department of Health, 1996), and the experiments complied with the current laws of Israel. The Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem approved the protocol.

Experimental procedure

The backs of haired guinea pigs were shaved 24 h prior to the experiment. The animals were anesthetized by intraperitoneal injection of 30 mg/kg sodium pentobarbital. The backs were cleaned with wet soft white paper and allowed to dry. Six sites on each back, three on each side, were exposed to 500 μg mechlorethamine (HN2, 10 μl of 50 mg/ml ethanol). A thin layer of 40 mg 10% povidone iodine (PI) ointment (Fisher Pharmaceutical, Tel Aviv, Israel) was applied to the entire exposed area 15 or 120 min after the HN2 exposure. Animals were killed (100 mg/kg pentobarbital sodium i.p.) 3, 24, 48 or 72 h after HN2 exposure. Skin area of 0.5×0.5 cm was removed and kept at -20°C until analysis.

Determination of collagenolytic activity

The collagenolytic activity was determined on a gelatin-impregnated (1 mg/ml; Difco, Detroit, Mich., USA) 8% sodium dodecyl sulfate-polyacrylamide gel, as previously described (Singh et al. 1995, 1997), with minor modifications. Briefly, samples were homogenized in developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl_2 , 0.02% w/v Brij-35 at pH 7.6) and centrifuged for 5 min at 14,000 g. The metalloproteinases were separated on the substrate-impregnated gels under non-reducing conditions, followed by 30-min incubation in 2.5% Triton X-100 (BDH, Poole, UK). The gels were then incubated for 16 h at 37°C in developing buffer to allow the separated proteinases to digest the gelatin. At the end of the incubation period,

the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, Calif., USA) in methanol:acetic acid:water (30:10:60). Since gelatin was degraded at sites of proteolytic activity, the bands appeared as colorless areas in the stained gel. The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A; Sunnyvale, Calif., USA).

Results

The effect of HN2 and iodine treatment on the collagenolytic activity is shown in Fig. 1a–c. All three collagenase activities were elevated after irritant exposure. The most pronounced effects were observed in matrix metallopro-

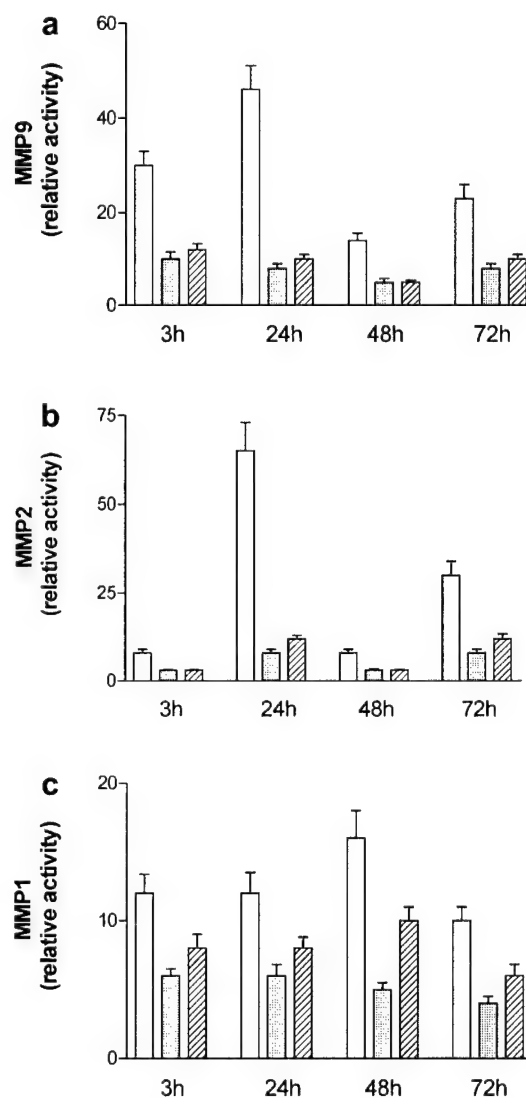


Fig. 1a–c. Effect of povidone iodine (PI) on mechlorethamine (HN2)-induced skin collagenolytic activity. Guinea pigs were topically exposed to HN2 (open bars); PI was applied 15 (dotted bars) and 120 min (hatched bars) after exposure. Skin specimens, collected 3, 24, 48 and 72 h after HN2 application, were analyzed for three types of collagenolytic activity, metalloproteinases MMP9 (a), MMP2 (b) and MMP1 (c). Results are expressed as the relative activity between that measured in treated and/or exposed skin and that of control tissue. Each column is the mean \pm SEM of three skin samples

teinase-9 (MMP-9, Fig. 1a) and MMP-2 (Fig. 1b) activities. The former was increased by 30, 46, 12 and 23% after 3, 24, 48 and 72 h of exposure, respectively, whereas the latter was elevated by 8, 65, 8 and 30%, respectively. Interestingly, PI treatment reduced the HN2-induced collagenolytic activity in all experimental conditions tested. Skin analyzed 3 h after HN2 exposure and treated with PI 15 or 120 min after the exposure, showed that the MMP-9 activity decreased by 67% and 60%, respectively. Reduction after PI treatment was also observed for MMP-2 (Fig. 1b) and MMP-1 (Fig. 1b) activities, albeit to a lesser extent than that observed for MMP-9. A stronger effect of PI treatment given 15 min after HN2 exposure was observed in skin analyzed 24 h after the exposure, i.e. decreases of 83% and 88% in MMP-9 and MMP-2 activities, respectively. Similar findings were observed with an interval of 120 min between HN2 exposure and PI treatment. A much weaker effect was observed for the MMP-1 activity. A similar trend of PI-induced reduction in the three types of collagenase activity was found in skin analyzed 48 and 72 h after exposure.

Discussion

The present findings demonstrate the strong inhibitory activity of PI, or its active ingredient iodine, on three types of collagenase. Since proteolytic activity is involved in inflammatory processes (Rikimaru et al. 1991; Woessner et al. 1990), which lead to skin lesion and necrosis mainly through the separation of the dermo-epidermal junction, it is reasonable to assume that the protective effect of iodine may stem, in part, from the reduced skin collagenase activity. This decreased activity can be a direct outcome of the action of iodine on the enzyme by oxidation of sulfhydryl residues in the protein, resulting in a conformational alteration that blocks its activity. An additional explanation for the iodine-induced decrease in collagenase activity may be an induction of an endogenous proteinase inhibitor that is capable of blocking the enzymatic activity. A further possibility is that iodine is acting at the transcriptional or translational levels of the collagenase genes, such that inhibition of enzyme synthesis is the cause for the reduced activity. Whatever the reason for the reduced collagenolytic activity of iodine, its beneficial effect against skin lesions has been proved in both experimental animals (Wormser et al. 1997, 2000a, 2000b) and humans (Wormser 1998). Future studies may involve synthetic proteinase inhibitors as a countermeasure against heat and chemical irritants.

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Genotoxicity study of a new tetraalkylammonium derivative of 6-methyluracil (agent No. 547)

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Abstract Agent No. 547 (1,3-bis[ω -(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide), a newly synthesized inhibitor of mammalian-specific acetylcholinesterase (EC 3.1.1.7) was investigated for genotoxicity using the DNA-repair test, Ames test and in vivo micronucleus test with mouse peripheral blood erythrocytes. Agent No. 547 did not cause significant changes in growth of repair-deficient *Escherichia coli* tester strains. The compound was non-mutagenic in *Salmonella typhimurium* strains TA98 and TA100 with and without rat microsomal activation mixture. However, we observed a marked increase in number of His⁺ revertants for both tester strains in preincubation assays. The results obtained in the micronucleus test indicate that agent No. 547 possesses significant clastogenic activity. At the high dose tested (0.5 mg/kg), the compound induced a seven-fold increase in the number of micronuclei over the spontaneous background 48 h after treatment. The results suggest that further work should be promoted to identify the metabolic pathways involved in genotoxicity of agent No. 547 in mammalian cells and to evaluate the real risk of its exposure.

Keywords 1,3-Bis[ω -(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide · Acetylcholinesterase inhibitor · Genotoxicity

Introduction

Synthetic inhibitors of cholinesterases are subjected to a considerable number of studies focused on the production of new drugs and pesticides. Recently a new class of highly effective and irreversible type inhibitors of mammalian-specific acetylcholinesterase (AChE; EC 3.1.1.7), which possess unique biochemical and toxic properties has been found among tetraalkylammonium derivatives of 6-methyluracil (Reznik et al. 1998).

1,3-Bis[ω -(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide (agent No. 547), one of the most active derivatives of 6-methyluracil, demonstrates at extremely low doses (0.1–1.0 nM) an irreversible and progressive type of inhibition of AChE from bovine erythrocytes without forming covalent bonds with the enzyme. The process can be characterized by a bimolecular rate constant ($k^0 = 3.5 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) (Reznik et al. 1998).

Despite effective interaction with AChE in vitro, agent No 547 and some of its analogues show a very low level of acute toxicity; the LD₅₀ for rats is 1.0–2.0 mg/kg body weight (Zobov et al. 1998).

Taking into account prospective applications of agent No. 547 in medicine and agriculture, it is necessary to evaluate the possible hazardous genetic endpoints of exposure to this chemical. In this connection, we designed studies to examine the genotoxic potency of the compound using endpoints of DNA-damage (DNA-repair test), mutagenicity (Ames test) and clastogenicity (micronucleus test).

Materials and methods

Chemicals

Agent No. 547, 1,3-bis[ω -(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide, was obtained from Arbuzov Institute of Organic and Physical Chemistry, Russian Academy of Science, Kazan, Russia. The chemical structure of agent No. 547 (Fig. 1) has been confirmed by mass spectrometry,

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Safety Evaluation — Pharmaceutical

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Protective Effect of Topical Iodine Preparations Upon Heat-Induced and Hydrofluoric Acid-Induced Skin Lesions

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ABSTRACT

In this study, the protective prophylactic and post-exposure effects of novel topical iodine preparations were demonstrated upon heat- and hydrofluoric acid-induced skin lesions in the haired guinea pig. Prophylactic treatment of thermal burns with a liquid iodine preparation resulted in statistically significant reductions of 39% and 30%, respectively, in acute inflammation and hemorrhage-microscopic dermal parameters indicative of acute tissue damage. A clear trend of iodine-induced reduction in dermal necrosis occurred, and the epidermal healing markers, acanthosis and hyperkeratosis, were increased. Post-exposure treatment of thermal burns with an iodine ointment preparation immediately after occurrence also conferred significant therapeutic reduction in parameters of tissue damage such as epidermal ulceration (87%), acute inflammation (58%), and hemorrhage (30%). Gross pathological evaluation showed that prophylactic and postexposure treatments with the liquid iodine preparation significantly reduced the heat-induced ulceration area by 97% and 65%, respectively. In addition, immediate treatment with an ointment iodine formulation significantly decreased the ulceration area by 98%; its tetraglycol vehicle also had a beneficial effect. Postexposure treatment with the iodine ointment proved efficacious upon hydrofluoric acid-induced skin burns. We observed statistically significant reductions of 76% and 68% in ulceration areas at intervals of 5 and 10 minutes between exposure and treatment, whereas a weaker effect was observed at a longer time interval of 15 minutes. Our findings suggest the therapeutic usage of these newly developed iodine preparations for thermally induced and hydrofluoric acid-induced skin burns.

Keywords. Chemical exposure; dermal toxicity.

INTRODUCTION

Thermal burns remain the major cause of traumatic injury world-wide, affecting all ages (18, 23). The current treatment of heat-induced skin wounds includes surgical procedures such as decompressive escharotomy with transplantation and burn dressing with topical antiseptics (9). Prehospital care, however, is of critical importance in reducing the severity of burn injury. The usual recommendation is to apply, as soon as possible, cool fresh water for at least 10 to 15 min (20); however, the danger of hypothermia precludes cooling of large thermal burns, especially in pediatric or elderly patients (9). Moreover, the gelatinous impregnated blankets designed to transfer heat from the wound to the dressing should be avoided when treating infants or young children after extensive burns, and in cases of unconsciousness and shock (9).

Iodine and its related compounds such as povidone-iodine are widely used as topical antiseptic agents (22). Recently, a newly developed topical iodine preparation was shown to

possess potent antidotal activity against mustard gas-induced skin lesions within 30 min following exposure (33). The iodine preparation was also effective against other alkylating agents, such as iodoacetate, cantharidine, and divinylsulfone (31, 32). These findings led us to the supposition that the anti-irritating property of iodine may be a more generalized effect (30). The present investigation tested the protective effect of newly developed iodine formulations upon both thermal and acidic burns in a controlled experimental system. To broaden the testing of the spectrum of protective iodine activity, we evaluated its effect upon hydrofluoric acid (HF), a strong skin irritant (4, 5, 27, 28) used in various industries (1).

MATERIALS AND METHODS

Animals: Dunkin Hartley male guinea pigs (600–800 g) were supplied by Harlan Laboratories Breeding Center, Ein Karem, Jerusalem, Israel. Following an acclimatization period of 7 days to ensure their suitability for the study, animals were randomly assigned to experimental groups. Test animals were kept within a limited-access rodent facility with environmental conditions set to a temperature of $20 \pm 2^\circ\text{C}$, a humidity of 30–70%, and a 12-hour light/12-hour dark cycle. Temperature and relative humidity were monitored

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daily. The animals were housed in filter-covered polypropylene cages with solid bottoms covered with wood shavings as bedding material. Animals were provided ad libitum access to a commercial guinea pig diet (Kofolk 19520, Netanya, Israel), and drinking water was supplied to each cage via propylene bottles with stainless steel sipper tubes. All procedures, maintenance, and treatment of the animals were in accordance with the principles of humane treatment published by the National Academy of Sciences. The Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem approved the protocol.

Iodine Formulations: The preparations tested were: formulation A, the ointment formulation—2% iodine, 50% tetraglycol (TG), 10% polyvinylpyrrolidone, 38% H₂O; formulation B, the liquid formulation—2% iodine, 98% TG; formulation A noniodine ointment vehicle—50% TG, 10% polyvinylpyrrolidone, 40% H₂O; and formulation B noniodine liquid vehicle—100% TG.

Experimental Procedure—Heat Burns: Backs of haired guinea pigs were shaved 24 hours prior to the experiment to minimize the effect of shaving. The animals were anesthetized by 30 mg/kg sodium pentobarbital IP. Backs were cleaned with wet, soft white paper and allowed to dry. Six sites on each back, 3 on each side, were exposed to the heat source (75°C water). Three exposure sites of each animal were treated with iodine, while the other 3 sites remained untreated. Heat exposure was accomplished using the well system. Wells were constructed on the skin by cutting a plastic tube cover (inner diameter of 1.7 cm) to form an open-ended cylindrical well. A thin layer of commercial silicon sealing ointment was applied to the edge of the well attached to the back of the animal so that liquid inside did not leak out. Then, 1 ml of 75°C water was applied into each well; 10 seconds later the liquid was aspirated and the exposed area immediately treated with iodine.

Three types of topical iodine treatments were tested: a) 1 ml of liquid iodine formulation B was placed in the well, b) after aspiration of hot water the well was removed and the skin was applied with 3 gauze-pad layers (2 × 2 cm) soaked in formulation B, c) following well removal a thin-layer of formulation A iodine ointment (40 mg) or its vehicle was applied. In all types of treatment iodine and its vehicle were removed after 2 hours. Anesthesia was maintained until the end of treatment by IP injections of 15 mg/kg sodium pentobarbital whenever needed. In an additional series of experiments the skin was prophylactically treated with iodine by placement of 1 ml of liquid iodine (formulation B) or its vehicle into the well for 2 hours. The liquid was then aspirated, and the placement of 1 ml of 75°C water into each well for 10 seconds was followed by water and well removal. Animals were sacrificed 4 days after treatment using 100 mg/kg sodium pentobarbital IP. Their backs were photographed alongside a ruler using a Kodak 260 digital camera. The ulceration area of each exposure site was assessed grossly by multiplying the length and width of the ulcerated area. Skin specimens were removed, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5–6 μ m, and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

Histopathological Evaluation: Each skin section was evaluated without knowledge of the treatment group and scored for histopathological changes. The reactive and inflammatory changes in the epidermis and dermis were assigned a severity grade of 0–4 representing unremarkable, minimal, mild, moderate, and marked changes, respectively. Epidermal parameters included subepidermal microblister formation, ulceration, necrosis, crust formation, hyperkeratosis, and acanthosis (epidermal hyperplasia). Dermal parameters included hemorrhage, inflammation (acute and subacute), necrosis, and fibrosis. In addition, the areas of epidermal acanthosis were estimated using grades of 0–6 as follows: 0—no acanthosis; 1—less than $\frac{1}{3}$ of the epidermal area acanthotic, the remainder necrotic; 2—approximately $\frac{1}{3}$ acanthotic, the remainder necrotic; 3—approximately $\frac{1}{2}$ acanthotic, the remainder necrotic; 4—more than $\frac{1}{2}$ but less than $\frac{2}{3}$ acanthotic, the remainder necrotic; 5—more than $\frac{2}{3}$ acanthotic, the remainder necrotic; 6—diffuse acanthosis with entire epidermal area acanthotic.

Quantitative Evaluation: To quantify the protective effect of the iodine formulations upon heat burns, we evaluated 27 skin specimens exposed to hot water only, 9 treated immediately after exposure with iodine (formulation A), and an additional 9 treated prophylactically with iodine (formulation B) before exposure. Skin specimens were collected 4 days after treatment. Results are expressed as the mean \pm SEM using the nonparametric Mann-Whitney U-test for statistical comparison of the effects of prophylactic and postexposure treatments of iodine on heat-induced skin burns.

Experimental Procedure—Hydrofluoric Acid-Induced Burns: Guinea pigs were prepared and anesthetized as described for heat burns. Six sites on each back, 3 on each side, were exposed to 10 μ l 60% hydrofluoric acid (HF). Three sites on each animal were treated with 40 mg of formulation A, the iodine ointment, at 5, 10, and 15 minutes after exposure. Anesthesia was maintained for 3 hours after treatment. Animals were sacrificed, photographed, and evaluated as described for heat burns.

RESULTS

Thermal Burns—Gross Pathological Findings: The macroscopic appearance of guinea pig skin exposed to 75°C water for 10 seconds and immediately thereafter treated topically with liquid iodine (formulation B, Figure 1) demonstrates the therapeutic effect of iodine in all these 3 treated sites when compared with the control sites. Evaluation of a series of experiments revealed statistically significant reductions of the ulceration area in the iodine-treated sites (Figure 2). Postexposure treatment with a well or gauze pad containing formulation B, liquid iodine, reduced the ulceration area by 65% and 66%, respectively. Prophylactic treatment with the liquid was more effective and resulted in a 97% decrease in the ulceration area, compared with 42% reduction with vehicle alone. A similar pronounced effect (98% decrease in ulceration area) was observed in sites that underwent postexposure treatment with formulation A, the iodine ointment. The formulation A vehicle also significantly reduced damage (80%) as compared to the control group but the incorporation of iodine yielded a more efficacious



FIGURE 1.—Macroscopic appearance of the protective effect of iodine upon heat-induced skin lesions. Six sites of shaved guinea pig back were exposed to 75°C water for 10 seconds; 3 left-side sites topically treated with iodine formulation B soaked in a gauze pad within 1 minute after exposure; photograph taken 2 days after treatment.

preparation (90% reduction) for postexposure treatment of thermal burns.

Thermal Burns—Histopathological Findings. The histopathological findings are exemplified in the micrographs in Figure 3 A–D and quantified for various treatment groups in Figures 4 and 5. Prophylactic treatment with the liquid iodine (formulation B) resulted in a statistically significant reduction of 39% and 30%, respectively, in acute inflammation and hemorrhage (Figure 5)—dermal parameters indicative of acute tissue damage. Although not statistically significant, a clear trend of iodine-induced reduction in dermal necrosis occurred as well. Moreover, the epidermal healing marker, acanthosis, was significantly increased by 45% (Figure 4), and the same trend, (50% increase) although not statistically significant, was observed with an additional healing marker, hyperkeratosis. These healing parameters constitute the most reliable histological proof of the effectiveness of iodine (Figure 3A and C). Postexposure treatment with iodine ointment (formulation A) also conferred a significant degree of therapy, albeit to a lesser extent than that observed in the prophylactic treatment with formulation B. Although the epider-

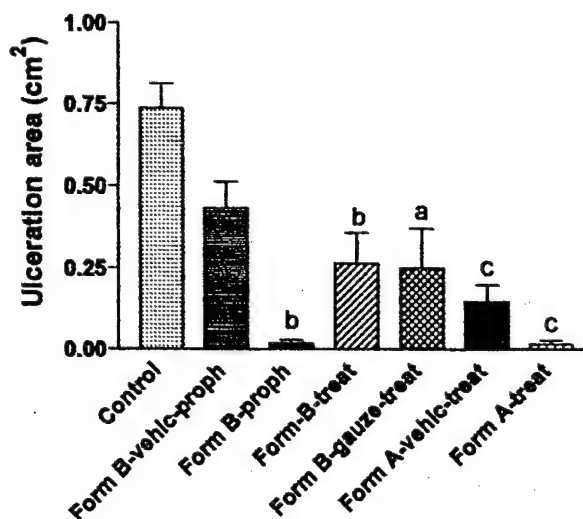


FIGURE 2.—Gross pathological quantification of the protective effect of iodine upon thermal burns. Each site on guinea pig back was exposed to 75°C water for 10 seconds; iodine treatment groups included immediate postexposure treatment with iodine formulation B (liquid in well, form B-treat, $n = 18$), prophylactic treatment with formulation B (in well, form B-proph, $n = 9$), postexposure treatment with gauze pad soaked in formulation B (form B-gauze-treat, $n = 9$), postexposure treatment with iodine formulation A (ointment, form A-treat, $n = 9$), postexposure treatment with formulation A vehicle (form A-vehic-treat, $n = 18$), prophylactic treatment with formulation B vehicle (form B-vehic-proph, $n = 18$); control group ($n = 81$) did not receive any treatment; ulcerated areas were measured 4 days following exposure. Results expressed as mean \pm SEM using Mann-Whitney U -test for statistical evaluation of differences between experimental groups; (a) $p < 0.05$, (b) $p < 0.005$, (c) $p < 0.001$ —comparison between each experimental group and the control; statistical significance ($p = 0.02$) observed between prophylactic treatment with formulation B and its vehicle.

mal healing markers were not elevated, some of the indicators of acute tissue damage, such as epidermal ulceration, and dermal acute inflammation and hemorrhage, were significantly reduced by 87%, 58%, and 30%, respectively (Figures 4 and 5).

Protective Effect of Iodine Against HF-Induced Skin Lesions: The therapeutic effect of iodine upon the acid burn is demonstrated macroscopically in Figure 6. None of the three sites treated with iodine developed ulceration, as compared to the untreated sites. Quantitative evaluations revealed statistically significant reductions of 76% and 68% in the ulceration areas at intervals of 5 and 10 minutes between exposure and treatment (Figure 7). Although not statistically significant, the beneficial effect of iodine was also observed at the time interval of 15 minutes with a 56% reduction in the ulceration area.

DISCUSSION

Our results demonstrate the protective effect of the newly developed TG-containing iodine formulation upon heat- and acid-induced skin lesions. The practical implications of these data for human clinical use are meaningful; if the skin

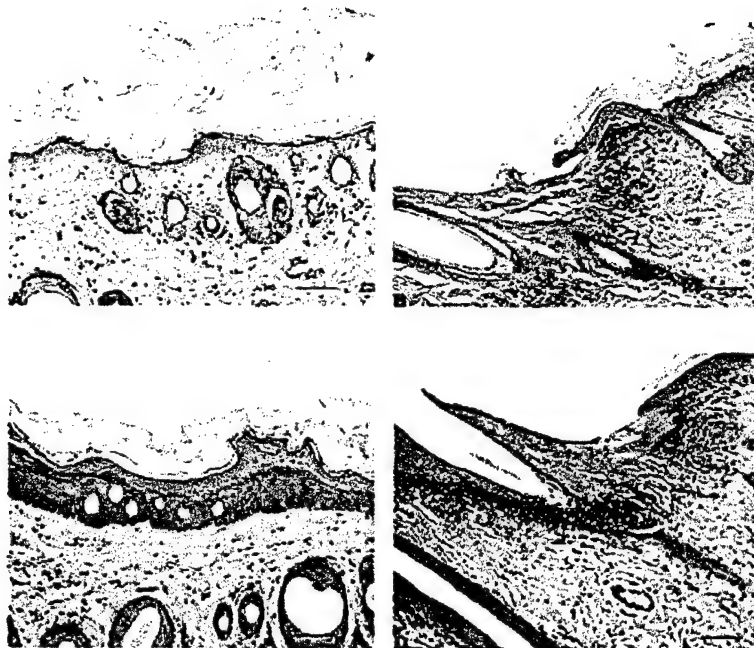


FIGURE 3.—Histopathology of skin exposed to hot water with and without iodine treatment. Photomicrographs of skin samples after exposure to 75°C water for 10 seconds. Epidermal necrosis implies death of cells in this layer. Ulceration implies loss of the epidermal layer. Encrustation implies formation of outer layer of solid matter formed by drying of exudate. A) Treatment of thermal burns with iodine ointment (formulation A) immediately after exposure; full thickness epidermal necrosis without ulceration apparent. H&E. Bar = 50 μ m. B) Thermal burns without iodine treatment after exposure; epidermal necrosis associated with ulceration and extensive acute dermal inflammation. H&E. Bar = 50 μ m. C) Treatment with liquid iodine (formulation B) before exposure to thermal burns; slight epidermal hyperplasia (acanthosis) and epidermal encrustation without ulceration. H&E. Bar = 50 μ m. D) No iodine treatment before exposure to thermal burn; epidermal necrosis associated with ulceration and extensive acute dermal inflammation. H&E. Bar = 50 μ m.

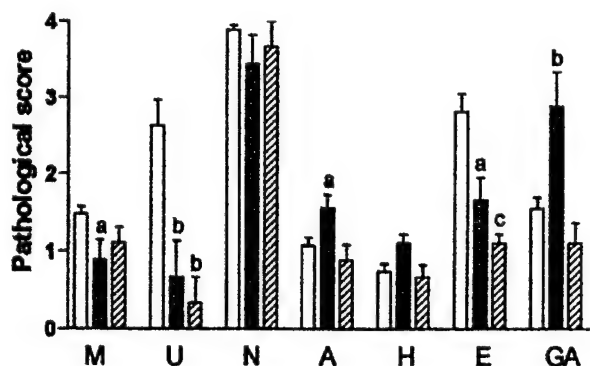


FIGURE 4.—Histopathological quantification of the protective effect of iodine upon thermal burns in the epidermal layer. Skin sections from guinea pigs exposed to 75°C water for 10 seconds, treated topically with iodine, and sacrificed 4 days after treatment; 7 epidermal parameters were determined: subepidermal microvesicles (M), ulceration (U), necrosis (N), acanthosis (A), hyperkeratosis (H), encrustation (E), and grade of area of epidermal acanthosis (GA). The following types of treatment were evaluated: skin exposed to heat stimulus only (open bars, $n = 27$), skin treated with liquid iodine (formulation B) for 2 hours prior to exposure to heat stimulus (dark bars, $n = 9$), skin exposed to heat stimulus and immediately afterward treated with iodine ointment (formulation A, hatched bars, $n = 9$). Results are expressed as mean \pm SEM using the Mann-Whitney U -test for statistical evaluation of the differences between controls (heat stimulus only) and postexposure and prophylactic treatments. (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$.

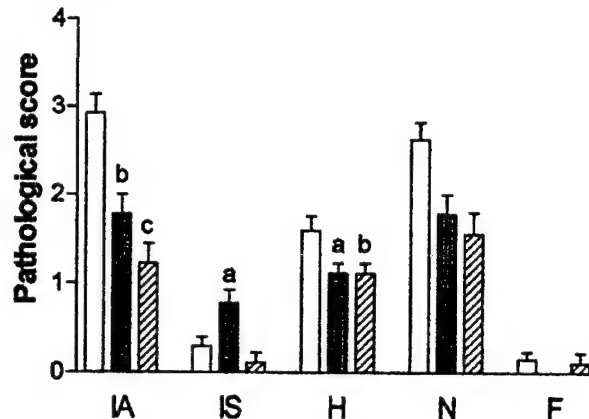


FIGURE 5.—Histopathological quantification of the protective effect of iodine against thermal burns in the dermal layer. Skin sections from guinea pigs exposed to 75°C water for 10 seconds, treated topically with iodine, and sacrificed 4 days after treatment; 5 dermal parameters were determined: acute inflammation (IA), subacute inflammation (IS), hemorrhage (H), necrosis (N), and fibrosis (F). The following types of treatment were evaluated: skin exposed to heat stimulus only (open bars, $n = 27$), skin treated with liquid iodine (formulation B) for 2 hours prior to exposure to heat stimulus (dark bars, $n = 9$), skin exposed to heat stimulus and immediately afterward treated with iodine ointment (formulation A, hatched bars, $n = 9$). Results are expressed as mean \pm SEM using the Mann-Whitney U -test for statistical evaluation of the differences between controls (heat stimulus only) and postexposure and prophylactic treatments. (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$.



FIGURE 6.—Macroscopic appearance of the protective effect of iodine on hydrofluoric acid-induced skin lesions. Six sites of shaved guinea pig back were exposed to 60% HF; 3 left-side sites were topically treated with iodine formulation A 5 minutes after exposure; photograph taken 3 days after treatment.

is treated early after exposure the injury can be greatly minimized. This investigation supports previous findings demonstrating the efficacy of a commercial povidone-iodine ointment in the treatment of human accidental heat burns (30). The previous human study reported experience with 20 burn cases; since that report 20 additional burn cases have been investigated by the author (personal communications). Immediate (within 2–3 minutes) topical treatment of heat burns with povidone-iodine resulted in prevention or significant reduction in skin damage (30). Although this report provided important clinical information, it was based on sporadic observations of patients and lacked human controls. Moreover, the commercially obtained povidone-iodine and other iodine preparations exhibited a low efficacy against thermal burns in the guinea pig (personal communications). Our present investigation demonstrates, by a controlled animal study, the therapeutic activity of the new type of iodine formulations upon heat- and acid-induced skin burns.

The main objective of our study was the development of efficacious iodine formulation producing optimal protection. The TG (solvent)-containing formulation was shown to pro-

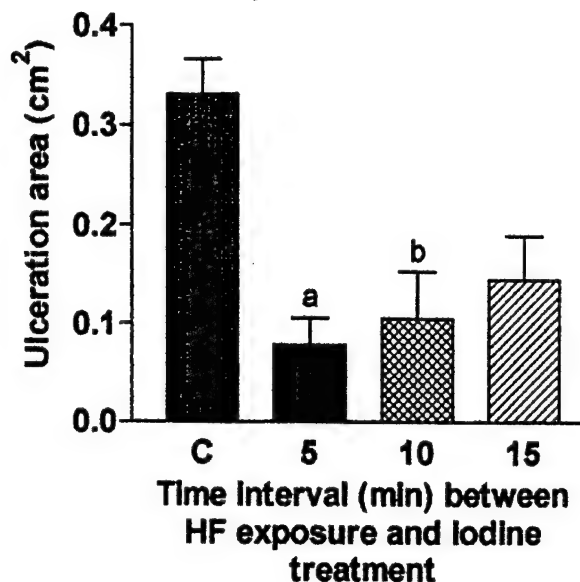


FIGURE 7.—Gross pathological quantification of the protective effect of iodine on HF-induced skin lesions. Each site on the guinea pig back was exposed to 10 μ l 60% HF; iodine ointment (formulation A) was applied 5 (n = 12), 10 (n = 6), and 15 (n = 9) minutes after HF exposure; controls (C, n = 27) did not receive iodine treatment; ulcerated areas were measured 3 days following exposure. Results are expressed as mean \pm SEM using the Kruskal-Wallis test and Dunnett's multiple comparison posttest for statistical evaluation of differences between experimental groups; (a) $p < 0.001$ for comparison between 5 minute interval and control; (b) $p < 0.05$ for comparison between 10 minute interval and control.

vide the most potent protection. As explained in our previous report (33), the superiority of this iodine formulation originates from its ability to dissolve molecular iodine in an aqueous environment. Molecular iodine (I_2) is practically water-insoluble unless iodide as sodium or potassium salt is present in the solution (22) to form water-soluble I_3^- . Other solvents such as ethanol may also dissolve iodine, but the presence of water precipitates it unless iodide is present to form the negatively charged I_3^- . The TG-containing formulation presumably preserves the uncharged molecular form of iodine (I_2), thus allowing its penetration into the skin and preserving its molecular form in the aqueous environment of the epidermal extracellular fluid so that it can exert its therapeutic activity. An additional advantage of iodine is that it manifested no skin-irritating effect, as confirmed by histological evaluation (data not shown).

Both prophylactic and postexposure iodine treatments manifested beneficial protective effects against thermal burns. However, the former was more potent than the latter using the liquid preparation, whereas the iodine ointment was also significantly efficacious in postexposure treatment (Figure 2). The protective effect of iodine upon mustard gas-induced skin toxicity was previously demonstrated (33) at intervals of 15 and 30 minutes between exposure and treatment. This time interval was significantly shortened while treating thermal burns in which iodine was applied shortly after exposure (30). This reduction coincides with the

relatively slow toxicokinetics of mustard gas. Mustard gas causes erythema 20–30 minutes following exposure, with edema and blisters occurring hours and tens of hours after exposure (29). In contrast, heat-induced burns develop much more rapidly. Iodine treatment of hydrofluoric acid-induced skin burns is effective within 10–15 minutes after exposure (Figure 7)—a time interval between that found for mustard gas- and heat-induced skin burns.

As shown in the present and previous studies, iodine was efficacious in all three types of tested burns. Differences in the effective interval between exposure and treatment may stem from the different timings of activation of pathological pathways inhibited by iodine. The hydrophobic and low molecular weight iodine molecule may be capable of penetrating cells and interfering with enzymatic processes leading to cellular damage and skin disruption.

Two possible mechanisms—inhibition of apoptosis or proteinase activity—may account for the protective effect of iodine therapy. Increasing evidence indicates that exposure to irritating factors is associated with the tendency of tissue to undergo programmed cell death. Heat shock-induced apoptotic cells were observed in cultured epidermal keratinocytes (16), HL 60 cells (12), and rat hair follicles (10). The apoptosis-related p53 protein was induced in heat-injured guinea pig skin (19). Apoptotic cells were also demonstrated after chemical stimuli. Apoptosis was observed, upon exposure to mustard gas and its derivatives, in cultured keratinocytes (24), endothelial cells (8) and cytoplasts (14), and thymocytes (13, 17). In vivo studies have shown the appearance of apoptotic cells in mustard gas-exposed skin of weanling pigs (26). The apoptotic process comprises a variety of biochemical reactions among which the activity of the cysteine proteinases, caspases, plays an important role (14, 15).

We hypothesize that iodine exerts its protective activity by inhibition of apoptotic processes, namely, by oxidizing sulfhydryl groups of either the active site of caspases or other functional proteins or peptides crucial for apoptosis. Apart from apoptosis-related proteinases, skin proteolytic activity may undergo activation upon receiving noxious stimuli. For instance, the cytotoxic effect of alkylating agents may be mediated by increased activity of proteinases (2, 3, 6, 7, 11, 21). Supportive findings have shown that proteinase (dispace) activity is experimentally employed for epidermal/dermal separation (25). We thus speculate that the protective effect of iodine may be derived from its ability to inhibit apoptosis or proteinase activity crucial for the evolution of skin damage. These hypotheses are under investigation.

Although the mechanism of iodine-induced therapy requires further investigations, our study demonstrates usefulness of the newly developed iodine formulations as potent topical preparations to treat and protect against skin lesions caused by hot water and hydrofluoric acid.

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Toxicokinetics and Metabolism

Skin toxicokinetics of mustard gas in the guinea pig: effect of hypochlorite and safety aspects

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Abstract. Sulfur mustard (SM, mustard gas) is a chemical warfare vesicant that rapidly penetrates the skin due to its hydrophobicity. This study measured the rate of SM disappearance from the skin after topical application of the vesicant. In both fur-covered and hairless animals, the remaining toxicant levels measured 60 min after exposure to undiluted SM were 0.6% and 0.3%, respectively, of the initially applied SM amount. However, SM concentration reached 0.4% of the initial dose 3 h following exposure in female fur-covered guinea pigs. SM quantities extracted from skin of male fur-covered and hairless guinea pigs immediately after 16 min of exposure to SM vapor were 12.2 and 21.8 µg, respectively; levels declined to 1.6 and 1.7 µg at 30 and 15 min following termination of exposure of male fur-covered and hairless guinea pigs, respectively. Three swabbing treatments of undiluted SM-exposed skin with gauze pads soaked in 0.5% hypochlorite caused 68% reduction in skin SM content. Similar findings were obtained when hypochlorite was replaced by water (64% reduction). SM content in the gauze pads was 59, 38 and 25 µg, respectively, for the first, second and third decontamination processes with water. No SM was detected in the gauze pads soaked with hypochlorite. In vitro studies showed that incubation of SM with 0.5% hypochlorite at a ratio of 10:1 (v/v) did not cause SM inactivation, whereas 4% hypochlorite reduced SM levels by 17%. However, at

a decontaminant:SM ratio of 1000:1, 0.5% and 4% hypochlorite reduced SM levels by 92% and 99%, respectively. These findings are important for health authorities and regulatory agencies in planning precautionary steps to be taken in case of emergency and in routine laboratory work.

Keywords. Mustard gas - Hypochlorite - Toxicokinetics - Topical exposure - Decontamination

Introduction

Sulfur mustard (SM), also termed mustard gas, is an oily liquid that easily penetrates the skin (Wormser 1991). Because of its devastating effect as a potent vesicant it has been employed as a chemical weapon in various conflicts during the twentieth century (Mellor et al. 1991). It functions as a powerful alkylator and a highly cytotoxic blisterogen in both humans and animals (Dacre and Goldman 1996; Filipek et al. 1997; Mellor et al. 1991; Mershon et al. 1990; Petralli and Oglesby-Megee 1997; Smith et al. 1995a, 1995b; Wormser 1991; Zhang and Monteiro-Riviere 1997). Skin exposed to SM develops erythema within 30 min to several hours after exposure, followed by edema, vesicle and blister formation, ulceration, necrosis and desquamation (Smith and Dunn 1991; Smith et al. 1995a, 1995b). Several therapeutic antidotes have been proposed for reducing SM-induced skin lesions including nucleophilic agents (Gross et al. 1997; Lindsay et al. 1997; Lindsay and Hambrook 1998; Wilde and Upshall 1994), arginine analogs (Sawyer 1998, 1999; Sawyer and Risk 2000; Sawyer et al. 1998), calcium channel blockers (Mazumder et al. 1998), niacinamide (Meier and Johnson 1992) and its combination with promethazine and indomethacin (Yourick et al. 1995). Recent studies have shown that povidone-iodine (Wormser et al. 1997, 2000a) and a novel iodine formulation (Wormser et al. 2000b) possess powerful antidote activity against SM-induced skin toxicity. The efficient use of any antidote requires better understanding of the mechanism of action of SM on the skin and, particularly, its toxicokinetic properties, namely, rate of penetration and reaction with skin components. Because of its hydrophobic characteristic, SM vapor rapidly penetrates human skin at a rate of 1-1.67 $\mu\text{g}/\text{cm}^2$ per min depending on time of exposure (Nagy et al. 1946). Recent studies with hairless guinea pigs using radiolabeled SM showed concentration- and time-dependent values in the range of 2 $\mu\text{g}/\text{cm}^2$ per min (Logan et al. 1999). In the isolated perfused skin flap model, peak radiolabel flux of SM occurred within 5 to 60 min after exposure to diluted SM (Riviere et al. 1995).

It is noteworthy, however, that the toxicokinetic characteristics of SM are most valuable from the practical point of view. In particular, the information about the rate of disappearance of SM from the skin is crucial, not only for safety precautions in laboratory handling of irritant-exposed animals but mainly for organizing the military and civilian emergency systems; namely, planning the decontamination procedures in terms of timing and methods, and precautionary measures to be taken during an en masse mustard gas attack. In the present study we have determined the kinetics of SM disappearance from the upper skin layers after exposure of male and female fur-covered guinea pigs and male hairless guinea pigs to undiluted SM and SM vapor. The effect of hypochlorite decontamination in vivo and in vitro was also determined.

Materials and methods

Precautionary steps

The work with SM was carried out according to the precautionary procedures of the Israeli Ministry of Labor and Welfare, Section of Labor Inspection, and the Department of Safety, The Hebrew University. All stages of synthesis and animal experimentation were performed in a continuously operating fume hood with an air flow of 125 feet/min. Investigators wore lab coats and masks. All contaminated glass and disposables were neutralized in a solution containing 1 N NaOH:ethanol (1:1).

The animals were kept in the hood until the end of the experiment. After being killed, the animals were packed in two thick nylon sacks and incinerated.

Animals

Male and female fur-covered guinea pigs (Dunken Hartley, 600-800 g) were supplied by Harlan Laboratories Breeding Center (Ein Karem, Jerusalem, Israel). Male hairless guinea pigs were purchased from Charles River Laboratories (Wilmington, Mass., USA). Following an acclimatization period of 7 and 14 days for the fur-covered and hairless guinea pigs, respectively, to ensure their suitability for the study, animals were randomly assigned to experimental groups. Test animals were maintained within a limited-access rodent facility with environmental conditions set to a temperature of $20 \pm 2^\circ\text{C}$, a humidity of 30-70% and a 12-h light/12-h dark cycle. Temperature and relative humidity were monitored daily. The animals were housed in filter-covered polypropylene cages with solid bottoms and provided with wood shavings as bedding material. Animals had ad libitum access to a commercial guinea pig diet (Kofolk 19520, Netanya, Israel), and drinking water was supplied to each cage via polypropylene bottles with stainless steel sipper tubes. All procedures, maintenance, and treatment of the animals were in accordance with the principles of humane treatment published by the National Academy of Sciences (US Department of Health 1996). In addition, the study protocol was approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem.

Experimental procedure

The backs of male and female fur-covered guinea pigs were shaved 24 h prior to the experiment. Both hairless and fur-covered guinea pigs were anesthetized by 30 mg/kg sodium pentobarbital i.p. The backs were cleaned with wet (water) soft white paper and allowed to dry. Six sites on each back, and three on each side, were exposed to $1 \mu\text{l}$ (1.27 mg) undiluted SM. At certain time intervals after exposure, the remaining SM was extracted from each exposure site by the following procedure. Wells were constructed over the back of the animals by adhering open-ended plastic cylindrical tubes (inner diameter of 1.7 cm) on the skin. A thin layer of commercial silicone sealing ointment was carefully applied on the edge of each cylindrical tube to be glued on the back of the animal so that the liquid inside did not leak out. Each well was exposed to SM followed by addition of 0.5 ml dichloromethane (Frutarom, Haifa, Israel), which was pipetted up and down three times, then collected into a 1.7-ml polypropylene tube. The dichloromethane samples were analyzed for SM content by gas chromatography/mass spectrometry (GC/MS). A repeated dichloromethane procedure did not detect SM in skin extract. Animals were killed using 100 mg/kg sodium pentobarbital i.p. In an attempt to validate this in situ extraction procedure of the living animal, skin samples of SM-exposed areas were taken from a guinea pig postmortem, cut into pieces (about $2 \times 2 \times 2 \text{ mm}$) and extracted with 0.5 ml

dichloromethane. The postmortem extracts were analyzed for SM, and the data were compared with the amounts of SM retrieved from in vivo skin extraction using the well system. The obtained postmortem results were similar to those obtained with the intact skin using the well system (data not shown).

In an additional series of experiments, guinea pigs were exposed to SM vapor by the following procedure. A circular paper disk (1-cm in diameter, Whatman no.1) was placed on the base of a cap (1 cm inner diameter) and soaked with SM (undiluted, $10 \mu\text{l}$). Five minutes later, the cap was attached on the center of a well by using a sealing ointment applied at the cap edges. The outer wells had been mounted on the backs of the animals as described above. The time of exposure to SM vapor was 16 min, after which the cap was removed and the exposed skin in each well was extracted with dichloromethane as described above. The extractions were performed immediately or after 15 and

30 min from the time of cap removal, i.e. from termination of vapor exposure. Results are the mean \pm SD of SM determinations of three skin samples.

Sulfur mustard analysis

SM concentrations in the dichloromethane extracts were quantified by GC/MS using appropriate standards. Specimens (5 μ l) were injected into a HP 5980 II gas chromatograph system (Hewlett Packard, Palo Alto, Calif., USA) equipped with an electron ionizing detector, HT1 30 m capillary column and HP 5971A MS detector. The elution method entailed starting at 100°C for 2 min then increasing the temperature at the rate of 10°C/min up to 200°C.

Determination of the effect of sodium hypochlorite on chemical inactivation of sulfur mustard in situ

Wells were constructed on the backs of pentobarbital-anesthetized guinea pigs as described above. The center of each well was exposed to SM (1 μ l undiluted liquid). Ten minutes later, the entire area of the skin in the well was swabbed with 3 layers of gauze pad (1.5 \times 1.5 cm) soaked in 0.5% sodium

↑ hypochlorite. This swabbing operation was repeated twice. For comparison, the same experiment was carried out with distilled water instead of sodium hypochlorite. The quantities of SM in the gauze pads and the skin enclosed in wells were extracted by 0.5 ml dichloromethane and analyzed by GC/MS.

Determination of the effect of sodium hypochlorite on chemical inactivation of sulfur mustard in vitro

SM (1 μ l) was incubated with 10 μ l or 1 ml 0.5% or 4% sodium hypochlorite. After 20 min at room temperature, SM was extracted with 1 ml dichloromethane by vigorous mixing. SM was determined by GC/MS.

Results

Sulfur mustard toxicokinetics in skin

1 The rate of disappearance of SM from the skin (2.3 cm² surface area in well) after exposure to 1 μ l (1.27 mg) of the undiluted irritant is illustrated in Fig. 1. As shown, a relatively fast decrease in skin SM content was observed in the male guinea pig in particular. In both fur-covered and hairless animals the SM levels, measured 60 min after exposure, were 0.6% and 0.3% (5.1 and 4.5 μ g), respectively, of the measured initially applied SM dose. However, relatively slower kinetics were observed in the female fur-covered guinea pig; an averaged quantity of 521 μ g SM (60.8% of measured initial dose) was determined after 60 min. Only 180 min after exposure to the SM did the skin content drop to as low as 0.4% of the measured initial dose. SM quantities extracted from skin of male fur-covered and hairless guinea pigs immediately after 16 min of exposure to SM vapor were 12.2 and 21.8 μ g, respectively (Fig. 2); the levels had declined to 1.6 and 1.7 μ g by 30 min (fur-covered guinea pigs) and 15 min (hairless guinea pigs) after termination of exposure, respectively.

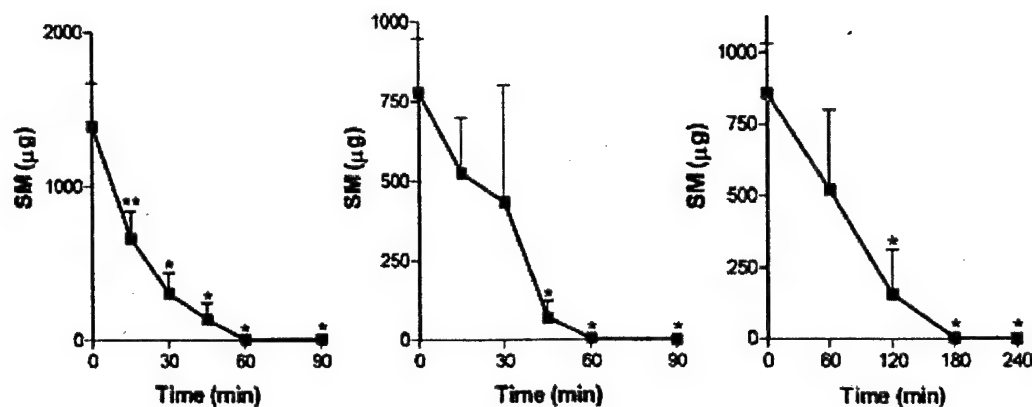


Fig. 1a-c. Skin toxicokinetics of undiluted sulfur mustard (SM). SM (1 μ l) was applied to the backs of shaved, anesthetized male hairless (a), male fur-covered (b) and female fur-covered (c) guinea pigs. At the indicated time intervals after exposure, SM was extracted from the skin by dichloromethane and analyzed by gas chromatography/mass spectrometry as described in Materials and methods section. Data for each time point are the mean \pm SD of three determinations. It is noteworthy that there was no full recovery of SM in the male fur-covered guinea pig skin (779 μ g instead of 1270 μ g), which may be due to a rapid reaction with skin components. Statistical significance of the difference between values obtained at each time point from that of zero time was evaluated by Student's *t*-test. ** $P < 0.05$, * $P < 0.02$

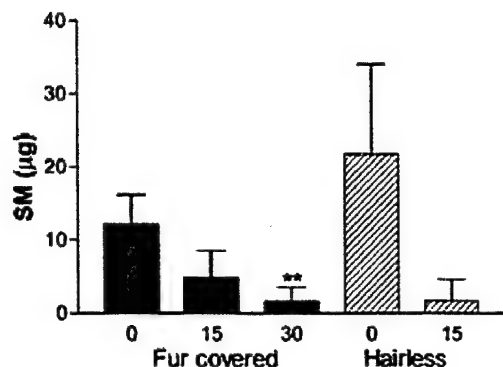


Fig. 2. Skin toxicokinetics of sulfur mustard (SM) vapor. Backs of shaved, anesthetized male fur-covered (~~solid bars~~) and hairless (*hatched bars*) guinea pigs were exposed to SM vapor for 16 min. At the indicated time intervals after termination of exposure (0 indicates within 1 min), SM was extracted from the skin by dichloromethane and analyzed by gas chromatography/mass spectrometry as described in Materials and methods section. Data for each time point are the mean \pm SD of three determinations. Statistical significance of the difference between value obtained at each time point from that of zero time was evaluated by Student's *t*-test. ***P* < 0.05

Decontamination of sulfur mustard by hypochlorite in vivo and in vitro

Topical treatment of SM-exposed skin with 0.5% hypochlorite (three successive swabbings with a gauze pads soaked in the decontaminant) caused 68% reduction in skin SM content (Fig. 3b), compared with the value determined when no swabbing had been performed. Similar findings were obtained when hypochlorite was replaced by water (64% reduction, Fig. 3b). The SM content in each of the three gauze pads was 59, 38 and 25 μ g for the first, second and third decontamination processes with water, respectively (Fig. 3a). No SM was detected in the gauze pads soaked with 0.5% hypochlorite solution. This implies an effective inactivation reaction by the decontaminant in the pad, in contrast to the skin in which it had a negligible effect. In vitro studies (Fig. 4) showed that incubation of SM with 0.5% hypochlorite solution at a ratio of 10:1 (v/v) did not cause SM inactivation, whereas 4% hypochlorite reduced SM levels by 17%. However, at a decontaminant:SM ratio of 1000:1, 0.5% and 4% hypochlorite reduced SM levels by 92% and 99%, respectively.

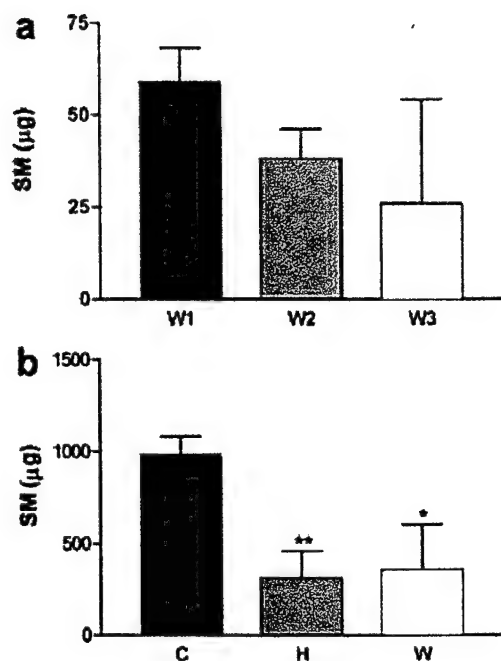


Fig. 3a,b. In situ skin decontamination of sulfur mustard (SM) by sodium hypochlorite and water. Shaved backs of fur-covered guinea pigs were exposed to SM (1 µl undiluted liquid). **a** Ten minutes later the skin was swabbed three times (*W1*, *W2*, *W3*) with three layers of gauze pad soaked in water. The pads were analyzed for total SM content. When water was replaced by 0.5% hypochlorite solution, no SM was detected in the pads. **b** Skin SM content was quantified by gas chromatography/mass spectrometry after hypochlorite (*H*) and water (*W*) swabbing and compared with control (no swabbing procedure, *C*). Exposed skin sites were immediately extracted with dichloromethane after swabbing as described in Materials and methods Section. Each bar represents the mean \pm SD of three determinations. Statistical significance of the difference between values obtained with hypochlorite and water treatment, and control was evaluated by Student's *t*-test. ** $P < 0.01$, * $P < 0.05$

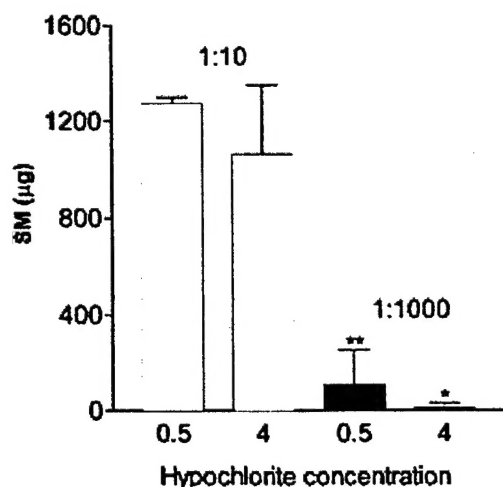


Fig. 4. Sulfur mustard (SM) decontamination by sodium hypochlorite in vitro. SM (1 µl undiluted) was incubated with 10 µl (1:10 v/v, ~~open bars~~) or 1 ml (1:1000 v/v, *solid bars*) sodium hypochlorite (0.5% or 4%). After 20 min of incubation at room temperature, SM was extracted and analyzed by gas chromatography/mass spectrometry as described in Materials and methods section. Each bar is the mean \pm SD of three determinations. Student's *t*-test was used for statistical evaluation of the difference between 1:10 and 1:1000 SM:hypochlorite ratio at hypochlorite concentrations of 0.5% and 4%. ***P*<0.005, **P*<0.05

Discussion

The present study describes the toxicokinetics of SM in guinea pig skin employing a new in vivo, non-invasive procedure of skin extraction in anesthetized animals. The methodology, which includes dichloromethane extraction of the corneal side of the skin, might have been questionable because the extraction process may not reach the SM in the deeper skin layer. However, the fact that an in vitro extraction of skin slices after in vivo exposure to SM gave similar recovery results to those obtained by the in vivo extraction procedure (explained in Materials and methods Section) indicates that the present procedure fully extracted the skin. Moreover, in a previous histochemical study (Cullumbine 1946), it was shown that free SM disappeared from the skin within 60 min after exposure of a fur-covered guinea pig to undiluted irritant, whereas in male humans the kinetics was shown to be faster, i.e. SM disappeared 30 min after exposure. This observation concurs well with our findings that demonstrate complete disappearance of SM 60 min after an exposure in both male fur-covered and hairless guinea pig skin. However, female fur-covered guinea pigs showed slower toxicokinetics, namely, SM disappeared 3 h after exposure. This might be associated with biochemical alterations in skin of females (Gloor et al. 1975) resulting in slower toxicokinetics, which affect the penetration rate and the alkylation of macromolecules. Interestingly, these sex differences were not reflected in the toxicodynamic properties of SM. No significant discrepancies in severity of skin damage were observed between male and female guinea pigs (data not shown), indicating that the rate of penetration does not play a crucial role in SM-induced skin toxicity. However, it is a crucial determinant for the decision-makers regarding decontamination procedures after mustard gas attack, as well as determining safety precautions in animal experimentation.

The rate of disappearance of SM from the skin after topical exposure is derived from a combination of certain factors. The properties of being a small molecule together with its hydrophobic nature (Gloor et al. 1975; Somani and Babu 1989) are important contributing factors to the rate of penetration of SM into the skin. Volatility is also a crucial determinant affecting its toxicokinetic and toxicodynamic properties (Nagy et al. 1946). Comparison between SM and chloroethyl ethyl sulfide vapor toxicity to human skin revealed that, despite being a mono-functional mustard, the latter showed powerful vesicating activity (Nagy et al. 1946). Presumably, due to its relatively high lipophilicity, chloroethyl ethyl sulfide penetrates human skin 5.1-fold faster than SM, resulting in equipotent vesicating activity to that of the bi-functional mustard in the vapor form (Nagy et al. 1946). Thus, the combination of lipophilicity, volatility and molecular size strongly affects rate of skin penetration, extent of alkylated macromolecules and, ultimately, the degree of skin lesion.

Additional important data associated with safety precautions are those describing the in situ and in vitro SM decontamination by sodium hypochlorite. Several laboratories dealing with SM research use hypochlorite for decontaminating skin of the living animal and also equipment such as tubes and disposables. The present study demonstrates that in situ skin decontamination by hypochlorite is equipotent to that of water, except for the fact that small quantities of SM left in the decontaminating pads containing water (Fig. 3a), whereas no SM was found in those pads soaked with hypochlorite. Since water does not inactivate SM (1 μ l irritant in 1 ml water for 1 h, followed by dichloromethane extraction; data not shown), these findings indicate that the reason for disappearance of SM from the skin after hypochlorite swabbing is due to some physical removal, e.g. adsorption, rather than chemical inactivation. These findings are in agreement with those of Gold et al. (1994) who showed that there was no difference between efficacy of hypochlorite solutions and water in the decontamination of wounds exposed to SM. Interestingly, topical treatment with iodine, an additional oxidizing agent, did not chemically inactivate SM (Wormser et al. 2000b). Nevertheless, it exerted pronounced protective activity against skin lesions induced by chemicals (Wormser et al. 1997, 2000b) and heat (Wormser 1998; Wormser et al. 2000a). This protective effect was not achieved by topical hypochlorite treatment (data not shown). The limitations of hypochlorite decontamination was also demonstrated in our in vitro studies, which showed that 4% or 0.5% hypochlorite neutralized SM only at an SM:hypochlorite ratio of 1:1000 (v/v), whereas 1:10 (v/v) did not significantly affect SM. This may stem from either weak reactivity of hypochlorite and/or low solubility of SM in the diluted hypochlorite solution. Therefore, from the present report one can reveal that incorporation of an organic solvent into the decontaminating preparation may enhance essential SM solubility, resulting in better efficacy of SM neutralization. As mentioned above, these finding should be considered by the military and occupational health authorities while planning precautionary steps to be taken in case of emergency and in routine laboratory work.

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